

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
4 January 2001 (04.01.2001)

PCT

(10) International Publication Number
WO 01/00823 A1

(51) International Patent Classification⁷: C12N 15/12,
C07K 14/72, A61K 38/17, C07K 16/28, G01N 33/53

(21) International Application Number: PCT/EP00/05981

(22) International Filing Date: 27 June 2000 (27.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
MI99A001433 29 June 1999 (29.06.1999) IT

(71) Applicant (for all designated States except US): EURO-
PEAN MOLECULAR BIOLOGY LABORATORY
[DE/DE]; Meyerstrasse 1, D-69117 Heidelberg (DE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): GANNON, Frank
[DE/IT]; Via Campo di Pile, I-67100 L'Aquila (IT).
DENER, Stefanie [DE/IT]; Via Campo di Pile, I-67100
L'Aquila (IT). FLOURIOT, Gilles [FR/IT]; Via Campo di
Pile, I-67100 L'Aquila (IT).

(74) Agents: MINOJA, Fabrizio et al.; Bianchetti Bracco Mi-
noja S.r.l., Via Rossini, 8, I-20122 Milan (IT).

(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE,
DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU,
ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS,
LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ,
PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT,
TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

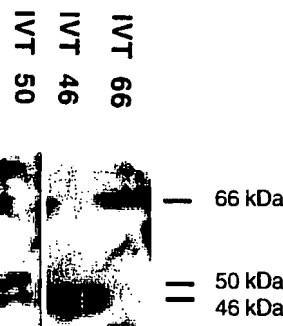
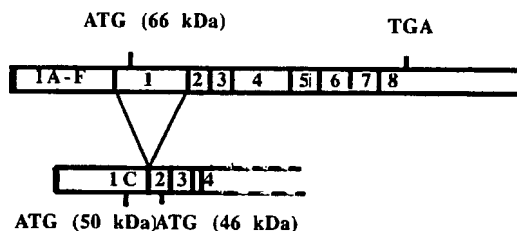
(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

- With international search report.
- Before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments.

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: NOVEL ISOFORMS OF THE HUMAN ESTROGEN RECEPTOR- α



(57) Abstract: There are disclosed polynucleotide sequences encoding novel hER- α splicing variants, the encoded polypeptides, the use thereof, a method for identifying molecules that modulate the activity of such polypeptides.



NOVEL ISOFORMS OF THE HUMAN ESTROGEN RECEPTOR- α

The present invention relates to novel isoforms of the human estrogen receptor- α (hER- α).

More particularly, the invention provides a polynucleotide sequence encoding newly identified hER- α splice variants, the encoded polypeptides, the use of such polynucleotides and polypeptides, a method for modulating the action of such polypeptides and a method for identifying molecules which modulate the actions of such polypeptides.

Estrogen receptors are important for normal development and various important physiological functions. Alterations in the expression of the ERs have been associated with pathogenesis of a number of diseases such as cancer, atherosclerosis and osteoporosis. To date, two estrogen receptors (ER- α and β), encoded by different genes, have been identified. These receptors belong to a large family of ligand-activated transcription factors, whose members regulate gene expression by interaction with cognate DNA sequences called responsive elements. ER proteins are composed of five structural domains, each having a unique function in ligand binding, gene promoter activation and activation with other factors of the general transcription apparatus. A transcriptional activation function 1 (AF 1) has been located in the A/B domain and functions as a hormone independent activator of transcription of estrogen-responsive genes such as pS2 and c-fos. In addition, it is also involved in growth factor interactions with ER- α signaling pathways. This domain has been hypothesized to activate target genes by associating with components of the core transcriptional machinery, such as co-activators and repressors. The DNA-binding domain (DBD) corresponds to region C. The D region allows ERs to alter conformation after ligand binding. It also contains nuclear localization signals as well as sequences required for dimerization of the receptor. The E region contains the ligand-binding domain (LBD), which is responsible for ligand-dependent activation of transcription. Additionally, a

hormone-dependent transcription activating function (AF2) is present in the LBD. Finally, the C-terminal F region, in addition to region E, influences AF2 via modulating the magnitude of gene transcription, but the relative contribution shows cell and promoter specificity.

5 It is known that the estrogen receptor- α gene in human (Flouriot et al.), chicken (Griffin et al.) and mouse, contains several promoters which are essential for the process of protein expression to be initiated. Differential promoter usage and alternative splicing events can lead to ER- α transcripts with differential transcription activity, which may help to potentiate the diverse action of estrogen through a single
10 gene. Human and chicken ER genes generate several mRNA variants (A-F hER- α mRNAs and A-D cER- α mRNAs) by alternative splicing of upstream exons to a common splice site upstream of the translation start site. Those transcripts differ in their 5'-untranslated region, therefore resulting in the translation of the same estrogen receptor protein of 66 kDa size.

15 First data that the standard ER- α protein is in fact not the only and main isoform expressed in all tissues were obtained from the chicken system. The existence of two forms of the chicken ER- α protein (cER- α), the previously reported receptor (cER- α form I) which has a size of 66 kDa and a new form, referred to as cER- α isoform II (61 kDa in size) has been discovered. This form is lacking the 42 amino acids at its N-
20 terminus present in the full length cER- α form I. Whereas the 66 kDa protein is the translation product of several cER- α mRNAs (A1-D), the cER- α isoform II is encoded by a new class of mRNA, which is transcribed in vivo from a specific promoter mapping the region of the previously assigned translation start site of the cER- α gene. S1 nuclease mapping analysis reveals that cER- α mRNA form II is liver
25 enriched. Both receptor forms I and II differ in their ability to modulate estrogen target gene expression in a promoter and cell-type specific manner. Whereas cER- α form I activates or represses in a strictly estrogen-dependent manner, the truncated form is characterized by a partial transactivation or repression activity in the absence

of its ligands. Sequence comparison of the N-terminal coding region of different vertebrate ER- α reveals a conservation of the translation start site of cER- α form II in other oviparous species (rainbow trout and xenopus leavis) but not in mammals (Griffin et al.).

5 Analyzing human tissues and cell lines, the inventors have now found that the human estrogen receptor- α can exist in two alternative forms, which are generated by alternative splicing of the receptor gene and have a molecular weight of 46 and 50 kDa.

First evidence of this existence came from RT-PCR and S1 nuclease mapping
10 analysis showing the presence of hER- α transcripts with a deletion of exon 1. Those shorter transcripts are generated by splicing events from two upstream exons 1C or 1E/F directly to a splice acceptor site of exon 2 at position +685. This position is upstream from an internal ATG located within exon 2 (+753/758) of the hER- α coding region. In vitro translation of these two novel hER- α mRNA splice variants
15 give rise to new ER- α proteins with the molecular size of 46 kDa (when exon 1E/F splices into exon 2) and 50 kDa (-missing the A/B domain -splicing of exon 1C to exon 2), respectively. Western blot analysis using hER antibodies mapping different domains of the receptor show the expression of both receptors (MW 66 kDa and 46 kDa) in MCF-7 cells.

20 The differential expression of hER- α variants plays an important role in human bone. PCR analysis and S1 analysis of primary human osteoblasts shows that the F-promoter is the predominantly used in bone cells. Southern blotting revealed two amplification products, a 1.8 kb fragment, corresponding to the full length receptor, when exon 1F splices to the common splice site at position +163, but also a shorter
25 PCR product. This product is due to a splicing event with the splice donor site located in exon 1E/F and a splice acceptor site located at position +753/758 in exon 2, which also leads to the expression of the hER protein variant of 46 kDa.

According to a first aspect, the invention provides a nucleic acid molecule

encoding a hER- α isoform having the sequence of SEQ ID No.: 1 or 2. The term "nucleic acid molecule", as used herein, comprises nucleic acid sequences which are degenerative to the above nucleic acid sequences as well as deletion, addition or insertion sequence variants. Furthermore, the invention relates to polynucleotides which hybridize to the herein above described sequences under stringent conditions.

The meaning of "stringent conditions" is well known to those skilled in the art and is explained for example in Sambrook, "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory, N.Y. (1989), or in Higgins and Hames (Eds) "Nucleic acid hybridization, a practical approach" IRL Press Oxford, Washington DC (1985).

Stringent conditions for hybridation or wash are for example 0.1 x SSC, 0.1% SDS at 65°C.

The present invention also relates to a vector comprising the above mentioned nucleic acid molecule. The vector may be a plasmid, cosmid, virus, bacteriophage or any other vector which is conventionally used in genetic engineering and which, besides the DNA sequence encoding the hER- α isoforms, may comprise additional control elements, like expression control elements, suitable for eukaryotic or prokaryotic expression, including a promoter, translation initiation codon, translation and insertion site and marker genes for the selection of said vector in a suitable host cell and under suitable conditions.

A further object of the invention is a host cell transfected or transformed with the vector of the invention or a non-human host carrying the vector of the invention. The host cell may be any prokaryotic or eukaryotic cell, whereas the non-human host is preferably a mammal.

According to a further aspect, the invention is directed to a protein isoform of hER- α having the amino acid sequence of SEQ ID No 3 or 4. The invention further comprises variants of the polypeptides of SEQ ID No 3 and 4, which differ from those polypeptides for conservative amino acid substitutions which involve the replacement

of a residue with another of similar characteristics, without altering the whole protein function.

Sequence variants have an identity of at least 60%, preferably 70%, more preferably 90% with the sequences of SEQ. ID No. 3 and 4.

5 A considerable amount of evidence indicates that the different isoforms of the estrogen receptor, namely the 46 kDa, the 50 kDa and the 66kDa human ER proteins, have different activities. In particular, a number of transfection experiments shows that the 46 kDa transcript and the 50 kDa protein act in a manner which is antagonistic to that of the Estrogen Receptor in the presence of Estradiol in certain tissues and with
10 certain promoters (Fig. 5 and 6). The experiments further demonstrate that the new protein isoform hER46 can transactivate promoters containing EREs in Hela cells in an E2-dependent manner whereas in HepG2 cells, activation of reporter gene transcription by this isoform is not detectable. Cotransfection experiments in HepG2 cells using plasmid constructs coding for the hER66 and hER46 demonstrated that
15 hER46 can act as a trans-dominant repressor of activation in a concentration-dependent manner (Fig.6).

The distribution of the isoforms in different tissues has been investigated using RT-PCR analysis or S1 nuclease mapping analysis (Fig. 3).

S1 analysis of bone using a specific probe which maps to this region shows that
20 splicing of exon 1E/F to exon 2 is detectable as a protected fragment in primary osteoblast RNA and in bone marrow RNA, where the signal consisted of approximately 50% of the total protected hER- α mRNA, but it is absent in SaOs RNA. Immunoprecipitation experiments demonstrate that both proteins are expressed in primary osteoblasts showing a ratio hER66 to hER46 of approximately 50%,
25 whereas in MCF-7 cells, the 46 kDa protein represents approximately 10% of total ER- α protein. The high expression level of the shorter ER protein variant compared to the full length receptor protein in bone cells might be explained by the additional expression of the 46 kDa isoform from the internal ATG due to leakiness in the start

of translation, as mentioned above. Since in osteoblasts both mechanisms can lead to the expression of a shorter protein of exactly the same size, it is difficult to distinguish whether one mechanism is more important in the formation of the hER46 protein than the other.

5 According to a further aspect, the invention provides antibodies that recognize and bind to the hER- α protein isoforms herein disclosed. Such antibodies include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, humanized, bispecific antibodies, or fragments such as Fv, Fab, Fab', or F(ab')₂.

10 Techniques for producing and processing polyclonal antibodies are known in the art and are described, for example, in Mayer and Walker, eds., "Immunochemical Methods in Cell and Molecular Biology", Academic Press, London (1987).

15 In a preferred embodiment, the antibodies can selectively distinguish the different isoforms of the receptor, and are raised to polypeptides corresponding to the N-terminal region of the protein. The antibodies of the invention are useful not only as modulator of receptor-estrogen interaction, but also in immunoassays to detect hER- α isoforms or in their purification.

20 A further object of the invention is a method for identifying molecules which bind to and activate or inhibit the hER- α isoforms. In a preferred embodiment, such a method is used to screen those compounds that selectively distinguish between the different receptor isoforms. The method will measure the ability of the test compound to block interaction of a ligand, preferably an estrogen, with the receptor, and in general comprises the step of a) incubating the receptor with the candidate molecule, b) adding a receptor ligand, c) determining whether the candidate molecule modulates the binding of the receptor to its ligand.

25 As an example, a cellular or a subcellular preparation expressing the receptor isoform could be incubated with labeled ligand in the presence of the candidate molecule. The ability of this molecule to block this interaction could then be measured. In a typical competition assay, for example, an appropriate radio-labelled

ligand and a potential agonist/antagonist may be added to a cell preparation containing the receptor under examination, or to a recombinant form of the receptor, under suitable conditions for a competitive inhibition assay, whereby the effectiveness of the candidate molecule to compete with the radiolabelled ligand for receptor binding is measured as the number of ligand molecules bound to the receptor. Alternatively, candidate molecules may be screened for their ability to augment or inhibit dimerization of the receptor isoform or to augment or inhibit the receptor from assuming the activated state, for example using a two hybrid system. Furthermore, molecules disrupting the ligand/receptor interaction may be identified using the recently described inverse two hybrid technique (Vidal M. et al., Proc. Natl. Aca. Sci. USA 93, 1996, 10315-10320). A functional assay may be designed in which the response to the test compound is measured in terms of DNA synthesis activation, for example by determining the expression of a reporter gene which is under the control of a nucleic acid sequence responsive to the specific receptor isoform.

The identification of molecules modulating the activity of the receptors herein disclosed allows to prepare therapeutical agents which are indicated for a number of important clinical conditions, including cancer, osteoporosis and other bone disorders, Alzheimer's disease as well as cardiovascular diseases.

Another embodiment of the present invention is the use of the coding sequences of hER- α isoforms for quantitatively determining the levels of corresponding mRNAs present in cell or tissue preparations and the subsequent use of these data in diagnosis and prognosis of diseases. Methods for quantitatively determining mRNA levels of hER- α isoforms include Northern blotting, in situ hybridization, nucleic acid hybridization, RT-PCR and S1 protection analysis. In a further embodiment, the invention comprises the quantitative determination of the protein isoforms expressed in different tissues by in vitro immunoassay of a tissue sample using specific polyclonal or monoclonal antibodies. The quantitative determination of differential expression of the receptor isoforms may be useful to provide a diagnosis or prognosis

of a particular pathological condition and to establish a proper therapeutic protocol. For example, it may be used to determine the nature of a cancer, the cancer progression in relation to treatments such as irradiation, chemotherapy or surgery, the metastatic nature of the cancer, or to monitor the response of the tumor to the therapeutic treatment and to provide a prognosis for the patient concerning the course of the disease.

The polypeptides, polynucleotides and agonist/inhibitors of the present invention may be used in combination with a suitable pharmaceutical carrier. Thus, the compositions of the invention comprise a therapeutically or prophylactically effective amount of the active substance, and a pharmaceutically acceptable carrier or excipient. As examples, carriers may be selected from saline, buffered saline, dextrose, water, glycerol, ethanol, and combination thereof. The pharmaceutical compositions may be administered in a convenient manner such as by the oral, topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes.

FIGURES

Fig. 1. Existence of novel liver enriched cER- α transcripts whose 5' ends are located downstream of the previously assigned translation start site

(A) Schematic representation of the cER- α transcripts. The original cER- α mRNA is encoded by eight exons labeled 1-8. The position of the two initiator methionines (ATG1- codon 1 and ATG2- codon 42) and the termination codon (TAA) are indicated. The division of the cER- α protein into six regions, A-F, together with the DNA - (region C) and hormone - (region E) binding domains are shown directly above the cDNA. The location of the splice acceptor site at +154 in exon 1A is also marked. Three alternative upstream 5' non coding exons (B, C and D) splice to this position giving rise to cER- α mRNA isoforms 1B-1D. Probe A (from -169 to + 318), B (from +1298 to +1678) and C (from +158 to +892) used for SI nuclease mapping are indicated. For primer extension analysis, a primer complementary to positions +360 to +572 of the cER- α cDNA was used. (B) Preliminary evidence for a new cER-

α mRNA (A2) using SI nuclease mapping analysis. 100 μ g of total RNA from laying hen oviduct and liver was hybridized to a labeled probe C and treated with SI nuclease. Yeast RNA was used as a negative control. The SI nuclease resistant hybrids were separated on a 4 % polyacrylamide gel adjacent to DNA molecular weight markers and free probe. The position of the two cER- α mRNA A2 transcription start sites are indicated on the right side of the figure. Also indicated are the relative positions of ATG1 and ATG2. (C) Mapping of the cER- α transcription initiation sites by primer extension. 50 μ g of total RNA from laying hen oviduct and liver was hybridized to the long primer, treated with reverse transcriptase and the extension products were separated on a sequencing gel. The transcription start sites of A1 and A2 cER- α mRNAs are indicated. Also indicated are the relative positions of ATG1 and ATG2. (D) The pattern of distribution and the relative levels of the two classes of cER- α transcripts, A1-D cER- α mRNAs and A2 cER- α mRNAs, were determined by SI nuclease mapping assays using total RNA from various sources as indicated at the top of each lane. M and F indicates male and female samples, respectively. Yeast RNA was used as negative control. Protected fragments are marked with arrows. The relative abundance of the two classes of cER- α mRNA transcripts are shown below each lane of Fig. 1D. The values were calculated from the densitometric scanning of the protected fragments obtained after SI nuclease analysis and expressed as the percentage of the total cER- α mRNA expressed in the oviduct. +/- indicates that a weak expression of cER- α mRNA was observed in a minority of the analyzed RNA samples.

Fig. 2. Two protein isoforms I and II of cER- α gene are produced *in vitro* and *in vivo*

(A) Schematic representation of the cDNAs inserted within the expression vectors pSG cER- α I, pSG cER- α II and HEO and encoding the cER- α form I, cER- α form II and hER- α proteins. The position of the two initiator methionines (ATG1- codon 1 and ATG2- codon 42) and the termination codon (TAA) are indicated. In the

expression vector pSG cER- α II, the cER- α sequences between nucleotides +158 and +308 were not included. Therefore, the sequences preceding ATG2 are noncoding. (B) pSG cER- α I, pSG cER- α II and HEO plasmids were *in vitro* transcribed and translated by rabbit reticulocyte lysate in the presence of [35 S] methionine. Translation products were resolved on a 10 % SDS-polyacrylamide gel and sized relative to the migration of prestained molecular size markers. (C) Nuclear protein extracts from chicken oviduct and liver were separated in parallel on the same gel and then subjected to immunoblotting with the H 222 antibody. Immunoreactive bands 66 and 61 KDa in size were visualized by ECL.

Fig. 3. Evidence for an alternative splicing event at exon 2 acceptor splice site of the hER- α gene.

Panel A: Experimental design for 1A hER- α mRNAs detection, indicating the location and the size of the single-stranded probe A and each protected fragment obtained after S1 digestion of probe/hER- α mRNA hybrids. Probe A (from +617 to +1538) was specific for normal hER-a transcripts (A/F hER- α mRNAs) but was also able to partially protect 1A hER- α mRNA isoforms up to the splice acceptor site position of exon 2. Open boxes indicate the unique (1A-1F) and common (1-8) exons encoding each normal hER- α mRNA isoforms. The position of the initiator methionine (ATG) and the termination codon (TGA) are indicated. The division of the hER-a protein into six regions, A-F, are shown directly above the cDNA.

Panel B: 30 μ g of total RNA from MCF7 cells and 30 μ g of Yeast RNA used as a negative control were hybridized to the labeled S1 probes A, treated with S1 nuclease and the resistant hybrids were separated on a sequencing gel as described in Experimental Procedure. The undigested probe is shown in a separate lane.

Fig. 4. Exon 1E is alternatively spliced to exon 1A or exon 2.

Panel A: Schematic representation of the RT-PCR experiment designed to identify 1A hER- α mRNAs. Open boxes indicate the unique (1A-1F) and the two first common (1A, 2) exons encoding each hER- α mRNA isoforms. Approximate locations

of primers are shown by short arrows. Primer IV, located in exon 2, was used to prime hER- α cDNA synthesis by revers transcriptase. Primers A1-F1 which are specific for each hER- α cDNA 5' region, were used in a round of PCR amplification with primer V which is nested to primer IV in exon 2. The oligonucleotide probes P1 and P2, respectively from exon 1A and 2, were used to confirm the specificity of the PCR products as well as the exon 1A deletion for some hER-a transcripts.

Panel B: The hER- α cDNA isoforms were amplified as described above, using total RNA from MCF7. PCR products were electrophoresed through an agarose gel and transferred by southern blot to a membrane which was then hybridized with the oligonucleotide probes P1 and P2 as described in Experimental Procedures. Positions of migration of the molecular size marker are shown on the left side of the figure.

Panel C: The sequence of the PCR products from lane E or F which did not hybridize to the oligonucleotide probe P1 but hybridized to P2 probe revealed that they contain the donor site of exon 1E joined to the acceptor site of exon 2.

Fig. 5. E/F and E/F 1A hER- α mRNA isoform distribution analysis.

Panel A: RT-PCR analysis. Open boxes indicate the unique (1E or 1F) and common (part of 1E and 1A-8) exons encoding E/F hER- α mRNA isoforms. Approximate locations of primer are shown by short arrows. Primer I, located in the 3' untranslated region of exon 8, was used to prime hER- α cDNA synthesis by reverse transcriptase, using total RNA from various sources as indicated at the top of each lane. Yeast total RNA was used as a negative control. Primer E/F1 which is specific for both E and F hER-a cDNA 5' regions (in the common part of exon 1E), was then used in a first round of PCR amplification with primer II which is nested to primer I in exon 8. A second round of PCR reaction was performed with a specific (E/F2) and common (III) nested primers. An oligonucleotide probe from exon 2 was used to confirm the specificity of the PCR products. Positions of migration of the molecular size markers are shown on the left side of the figure.

Panel B, C and D: S1 nuclease mapping analysis. The S1 nuclease mapping

assays of E/F and E/F 1A hER- α mRNA isoforms were performed as described in Materials and Methods, with the single-stranded probes F (panel B), F 1A (panel C) and E 1A (panel D) and using 30 μ g of total RNA from various sources as indicated at the top of each lane. Yeast total RNA was used as negative control. The location and the size of each single-stranded probe (F, F 1A and E 1A) and each protected fragment obtained after S1 digestion of the probe/hER- α mRNA hybrids are indicated. Each probe was specific for one hER- α transcript (for example, F 1A hER mRNA) but was also able to partially protect the other hER- α mRNA isoforms [for example, (-E/F 1A) hER mRNA] up to the splice site positions. The probes were designed to contain vector sequence in their extremity (denoted by the thinner black line) in order to discriminate between undigested probes (>) and specific protected fragments.

Fig. 6. E/F 1A hER- α mRNA isoforms encode a 46 kDa protein which lacks the A/B domain present in the 66 kDa form.

Panel A: Schematic representation of the cDNAs inserted within the expression vector pSG5 which gave rise to pSG hER- α 66 (HEO) and pSG hER- α 46. The position of the initiator methionine for the 66 kDa hER- α , the initiator methionine for the 46 kDa hER- α and the termination codon (TGA) are indicated. The division of the hER- α protein (66 kDa) into six regions, A-F, together with the DNA - (region C) and hormone - (region E) binding domains are shown directly above the cDNAs. Also showed are the epitopes recognized by the anti-hER antibodies HC20, H226 and H222, used in panel B. HC20 is a polyclonal antibody and H226 and H222 are monoclonal antibodies.

Panel B: pSG hER- α 66 and pSG hER- α 46 plasmids were in vitro transcribed and translated in rabbit reticulocyte lysate. The obtained translation products as well as 40 μ g of whole cell extracts from MCF7 (ER positive breast cancer cell line), MDA-MB-435 (ER negative breast cancer cell line) and HeLa (ER negative cell line) were resolved on a 10 % SDS-polyacrylamide gel and then subjected to immunoblotting with the HC20, H226 and H222 antibodies. Immunoreactive bands 66

and 46 kDa in size were visualized by ECL.

Fig. 7. The 66 and 46 kDa hER- α differ in their ability to modulate estrogen target gene expression.

HeLa and HepG2 cells were transiently transfected with 5 μ g of the reporter plasmid coll73(API)-LUC or (ERE)2-tk-LUC, together with 0.5 μ g of the expression vector pSG5, pSG hER-a 66 (HEO) or pSG hER- α 46. Cells were treated with or without estradiol (10^{-8} M) for 48 h before being assayed for luciferase activity. Results are expressed as a percentage of the reporter gene activity measured in presence of the expression vector pSG hER- α 66 and E2. Luciferase activities were normalized using the internal reference control EF-1a-CAT. Values correspond to the average of at least three separate transfection experiments \pm SEM.

Fig. 8. The 46 kDa hER- α is a dominant negative form in HepG2 cells, suppressing the activity of the 66 kDa hER- α .

HepG2 cells were transiently transfected with 5 μ g of the reporter plasmid (ERE)2-tk-LU(together with either 0.5 μ g of the expression vector pSG5, 0.5 μ g of pSG hER- α 46 or 0.5 μ g of pSG hER- α 66 (HEO) with increasing concentration of pSG hER- α 46 (0 to 4 μ g). Cells were treated with 10^{-8} M estradiol for 48 h before being assayed for luciferase activity. Results are expressed as a percentage of the reporter gene activity measured in presence of the expression vector pSG hER- α 66. Luciferase activities were normalized using the internal reference control EF- 1 a-CAT.

Fig. 9. Panel A: Immunoprecipitation of estrogen receptor proteins in human primary osteoblasts demonstrate the expression of a 66 kDa ER- α protein as a result of the translation start at position +233 in exon1. A second translational product of 46 kDa size results from a splicing event of an upstream exon (1F/E) directly to position +685 in exon 2, which is located upstream from a internal ATG (+753/758). The positions of the initiator methionine (ATG) and the termination codon (TGA) are indicated. An isoform with a molecular weight of 39 kDa which was also precipitated

using H222 monoclonal antibody has to be further characterized. In vitro transcribed and translated pSG5 expression vectors encoding either the full length hER- α (IVT66) or the 46 kDa isoform (IVT46) in the presence of 35-S methionine were used as controls.

5 Panel B: In vitro translation of 50 kDa protein isoform. Examination of exon 1C of the hER- α gene demonstrated the beginning of an open reading frame which is in frame to the remainder of the hER- α open reading frame when spliced to the acceptor site at position +685 in exon 2. In order to investigate, whether this in frame ATG could function as a translation initiation codon for a shorter hER- α protein isoform of
10 a predicted size of 50 kDa cDNA was inserted into pSG5 expression vector. Western blotting revealed that this splicing event leads to the expression of a hER- α protein of 50 kDa size as well as to the expression of the hER- α 46 protein isoform due to the leakiness in the translation initiation. IVT66 and IVT46 were used as controls.

EXPERIMENTAL PROCEDURES.

15 Cells and tissues

All cell lines were maintained in DMEM (Gibco, BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS, Gibco BRL), penicillin (100 U/ml) and streptomycin (100 μ g/ml) at 37°C in a 5% incubator. Human primary osteoblasts were isolated from bone tissues of patients that underwent hip or knee operations under
20 sterile conditions. The outgrowing osteoblast cells were cultivated in DMEM + 20% FCS, characterized by staining for alkaline phosphatase (AP kit, Sigma, Deisenhofen, Germany) and taken for experiments until passage 3.

RNA isolation and RT-PCR (Fig. 2)

25 Expression of different hER- α isoforms was examined using reverse transcription of RNA followed by PCR and Southern Blotting. Total RNA was isolated from MCF-7 cells using TRIzol reagent (Gibco, BRL) as described by the manufacturer. Reverse transcription was performed using 1 μ g of total RNA, an oligonucleotide primer (I) from exon 8, located within the 3' untranslated region (3'

UTR) of hER- α (5' TTGGCTAAAGTGGTGCATGATGAGG) instead of an oligo (dT) primer and 50 U of Expand reverse transcriptase (Roche Diagnostics, Mannheim, Germany) following the protocol of the supplier. Two microlitres of the reaction were then used for two rounds of 35 cycles PCR amplification. The 5' primers and nested
5 primers used for the amplification of hER isoforms A, B, C, E and F were:

A1 (5' CTCGCGTGTCTGGCGGGACAT SEQ ID No. 5) and

A2 (5' GCTGCGTCGCCTCTAACCTC SEQ ID No 6),

B1 (5' CTGGCCGTGAAACTCAGCCT SEQ ID No 7) and

B2 (5' ATCCAGCAGCGACGACAAGT SEQ ID No 8),

10 C1 (5' TCTCTCGGCCCTTGACTTCT SEQ ID No 9) and

C2 (5' CAAGCCCATGGAACATTTCTG SEQ ID No 10),

E1 (5' AGCCTCAAATATCTCCAAAATCT SEQ ID No 11) and

E2 (5'AATTATATTCTGTAGCTACCAAAGAAG SEQ ID No 12),

F1 (5' TTCTATAGCATAAGAAGACAG SEQ ID No 13) and

15 F2 (5' GAGTGATAATCTTC SEQ ID No 14), respectively. The 3' primer V (5'ATTATCTGAACCGTGTGGGAG SEQ ID No 15) was chosen within exon 2 of the hER- α gene. A nested primer IV (5' CGTGAAGTACGACATGTCTAC SEQ ID No 16) was selected upstream of primer V. the Expand Long Template PCR system (Roche Diagnostics) was used for amplification as recommended by the manufacturer.

20 Five microlitres of each reaction were analyzed on a 1% agarose gel.

Southern Blotting (Fig. 2)

After separation on agarose gels, PCR products were transferred to nylon membranes (Hybond N+, Amersham, Arlington Heights, IL) with 20x saline sodium citrate (SSC) as transfer solution. The membranes were incubated in a
25 prehybridization buffer containing 6x SSC, 5x Denhardt's solution, 0.05% sodium pyrophosphate, 100 μ g salmon sperm DNA, and 0.5% SDS at 37°C for 1h. then, the membranes were hybridized in 6xSSC, 1xDenhardt's solution, 0.05% sodium pyrophosphate, 100 μ g/ml yeast t-RNA with an oligonucleotide probe selected in exon

1 (1) and exon 2 (P2), respectively, which had been end-labeled using T4 polynucleotide kinase and [γ -32P] ATP (3000 Ci/mmol, Amersham)). The most stringent wash was carried out for 20 min at 55°C in 6xSSC, 0.05% sodium pyrophosphate. The specific PCR products were visualized by exposing the
5 membranes to an x-ray film.

S1 nuclease assay (Fig 1, Fig 3)

A modified S1 nuclease mapping procedure was followed as described by Flouriot et al. The method involves the use of biotinylated single-stranded DNA probes by extension from a specific primer by extension from a specific primer by the
10 T7 DNA polymerase in the presence of [α 32-P] deoxy-CTP (3000 Ci/mmol). These probes are then hybridized with the appropriate RNA sample and subjected to an S1 nuclease digestion. In order to prepare the templates for the different probes, RT-PCR reactions were performed. The PCR products were subcloned upstream of T7 primer and downstream of M13 reverse primer in the TA cloning vector pCRTM2.1
15 (Invitrogen, San Diego, CA). a PCR reaction was then performed using a biotinylated forward primer together with a reverse primer either from vector (M13) or hER- α coding region. Biotinylated PCR products were bound to streptavidin-coated magnetic beads (Dynal, Great Neck, NY) as recommended by the manufacturer and the nonbiotinylated DNA strands were removed using magnetic separation, 105 cpm
20 probe was coprecipitated with 30-100 μ g total RNA and then dissolved in 20 μ l hybridization buffer (80% formamide, 40 mM piperazine-N,N'-bis[2-ethane sulfonic acid] (Pipes, pH 6.4), 400 mM NaCl, 1 mM EDTA (pH 8)), denatured at 70°C for 10 min, and hybridized overnight at 55°C. The S1 digestions were carried out for 1h at
25 30°C and after precipitation the samples were separated on 4% polyacrylamide-urea gels.

ER- α expression vector preparation (fig 4, Fig. 7)

To create the expression vector pSGhER46, PCR was used to amplify cDNA of the hER coding region from position +519 up to +1788 using the plasmid pSG5HEO

(kindly provided by P. Chambon). In order to construct pSG5hER50, a specific primer (5'TCTCTCGGCCCTTGACTTCTGCCAAATTCAGATAATCGACGCCAGGG SEQ ID No 17) was designed, mapping the hER- α isoform C and extending directly into exon 2. The reverse primer started in exon 2 and extended into exon 1C (5' CCACCCTGGCGTCGATTATCTGAATTTGGCAGAAGTCAAGGGCCGAGAGA SEQ ID No 18). An additional PCR resulted in amplification of exon 1C directly linked to position +452 in exon 2 up to position +1788 in exon 8. The primers were designed to introduce BamHI restriction sites at the ends of the PCR products. The amplified fragments were directionally cloned into the polylinker of pSG5 expression plasmid downstream of SV40 promoter.

In vitro transcription and translation (FIG. 4, Fig. 7)

In vitro transcription and translation was accomplished with the TNT coupled Reticulocyte Lysate system from Promega (Madison , WI, USA) following the manufacturers protocol. The expression vector pSG5HEO, pSGhER50 and pSGhER46 were used as templated for transcription with T7 RNA polymerase followed by translation to generate human ER- α proteins (hER66, hER50 and hER46).

Western Blot analysis (Fig. 4, Fig 7B)

Total cell lysates (40 μ g) and five microlitres of the in vitro transcription and translation mix were run on a 10% SDS-PAGE gel as outlined by Laemmli (2) and electrotransferred to Optitran membrane (Schleicher & Schuell, Dassel, Germany). The membrane was blocked in TBST (10 mM Tris, pH 7.4, 0.5 M NaCl, and 0.5% Tween 20), containing 5% non fat dry milk powder. The membrane was incubated with primary antibody (1 μ g/ml) in TBST for 1h at room temperature. Primary antibodies used were rat monoclonal antibodies H222, directed against the hormone-binding domain of hER- α , and H226, directed against the A/B domain of hER- α (both kindly provided by G. Greene, University of Chicago) as well as a rabbit polyclonal antiserum HC20 (Santa Cruz Biotechnology, Santa Cruz, CA), which mapped both

isotopes. Incubation with secondary peroxidase-conjugated goat-anti rat (H222, H226) or peroxidase-coupled goat anti-rabbit (HC20) was performed under the same conditions. ER- α proteins were visualized by chemiluminescence using the ECL system from Amersham according to the manufacturers instructions.

5 **Immunoprecipitation (Fig. 7A)**

Human primary osteoblasts were cultivated in 15 cm TC-plated in DMEM + 10% FCS until subconfluency. After twice washing with PBS, the cells were incubated in methionin-free DMEM + 10% methionin-free FCS for 10 hours. Then, 1000 μ Ci radiolabeled Pro-mix (35S-methionin/35S-cystein-mix. Amersham) were
10 added per plate and the cells were incubated overnight at 37°C and 5% CO₂. After washing with cold PBS, the cells were harvested and lysed in 1 ml RIPA-buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) for 30 min at 4°C. The lysate and the in vitro transcribed and translated hER46 and hER66, which served as positive controls, were incubated with an unspecific
15 antibody for 1h (anti- α -Actin, 1 μ g/ml, Roche Diagnostics), followed by binding to 5% protein-A-sepharose (Pharmacia Biotech, Freiburg, Germany) and centrifuged for 5 min at 10000 rpm. Surnatants were incubated with a hER- α specific antibody (H222, 1 μ g/ml) and then linked to sepharose for 1h at 4°C. After extensive washes, Laemmli-buffer was added to the precipitates and the samples were boiled for 5 min.
20 After centrifugation, the supernatants were separated on a 10% PAGE gel, dried and then subjected to autoradiography.

Transient transfection (Fig. 5, Fig. 6)

For transfection experiments, cells were plated into 6 cm dishes (Nunc, Wiesbaden, Germany) at a density of 1×10^5 cells/plate and grown in DMEM media
25 supplemented with 10% FCS. After 3 days, the cells were washed with 1xPBS and the medium was replaced by phenol-red free DMEM + 2.5% charcoal stripped, E2-free FCS. After additional 24 hours, transient transfections were carried out using the calcium precipitate method. Transfections were performed using 5 μ g of luciferase

reporter plasmid containing estrogen responsive elements (EREtkLuc) (Fig. 5 and 6) or an AP-1 site (AP-1(coll73)-Luc) (Fig 5) together with 0.25 µg of a CAT plasmid as a correction for transfection efficiency and 0.5 µg of pSG₅ expression plasmid coding for hER66 (pSG₅HEO) or hER46 (pSGhER46). For competition experiments, 5 pSG₅HEO was kept at a concentration of 5.0 µg to 0.006 µg while contemporaneously pSGhER46 was added at increasing dilutions comprised in the range 4 µg – 0.006 µg. After overnight incubation, the transfection media was removed, the cells were washed twice with PBS, and 3 ml of phenol-red free DMEM supplemented with 2.5% charcoal stripped FCS and 10⁻¹⁰M estradiol were added. After 48 hours cells were 10 harvested and luciferase assays and CAT ELISAs were performed using commercial kits (Roche Diagnostics).

CLAIMS

1. A nucleic acid molecule encoding a hER- α isoform having the sequence SEQ ID No 1 or 2.
- 5 2. A nucleic acid molecule hybridizing to the nucleic acid molecule of claim 1 under stringent conditions.
3. A polypeptide encoded by the nucleic acid molecule of claim 1.
4. A polypeptide according to claim 3, having the sequence SEQ ID No 3 or 4.
5. A vector comprising the nucleic acid molecule of claim 1 or 2.
- 10 6. A host cell genetically engineered with the vector of claim 5.
7. An antibody that binds to the polypeptide of claim 3 or 4.
8. The antibody of claim 7, which is selected from the group consisting of a monoclonal antibody, a humanized antibody, a human antibody, a bispecific antibody, a chimeric antibody, and an antibody fragment such as Fv, Fab, Fab', or F(ab')₂.
- 15 9. A method for identifying molecules which bind to the hER- α isoforms, which comprises the steps of:
 - a) incubating the receptor with the candidate molecule,
 - b) adding a receptor ligand,
 - c) determining whether the candidate molecule modulates the binding of the
- 20 receptor to its ligand.
10. A pharmaceutical composition comprising a nucleic acid molecule of claim 1 or 2 or a polypeptide of claim 3 or 4, together with a pharmaceutically acceptable carrier.

A

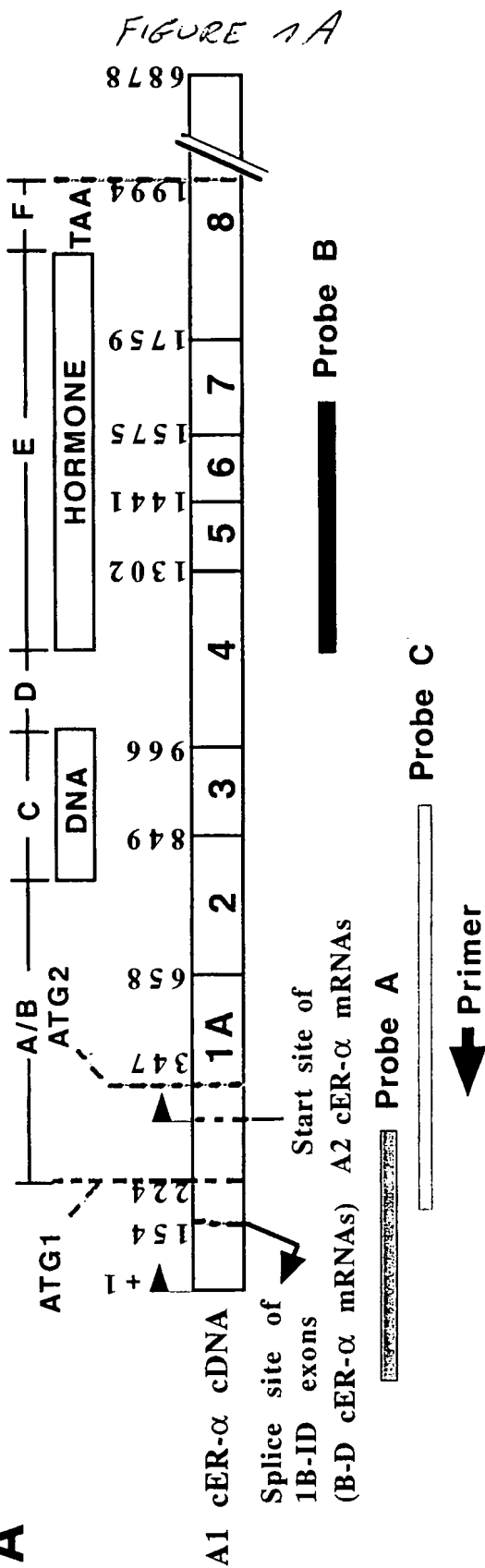


FIGURE 1B

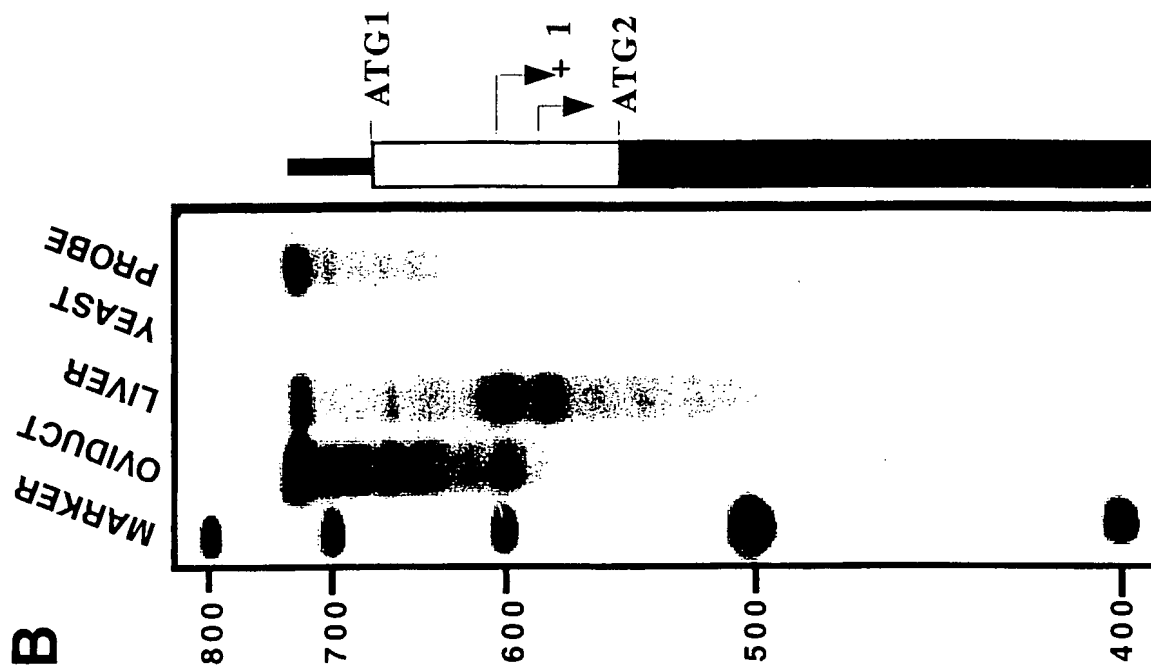


FIGURE 1C

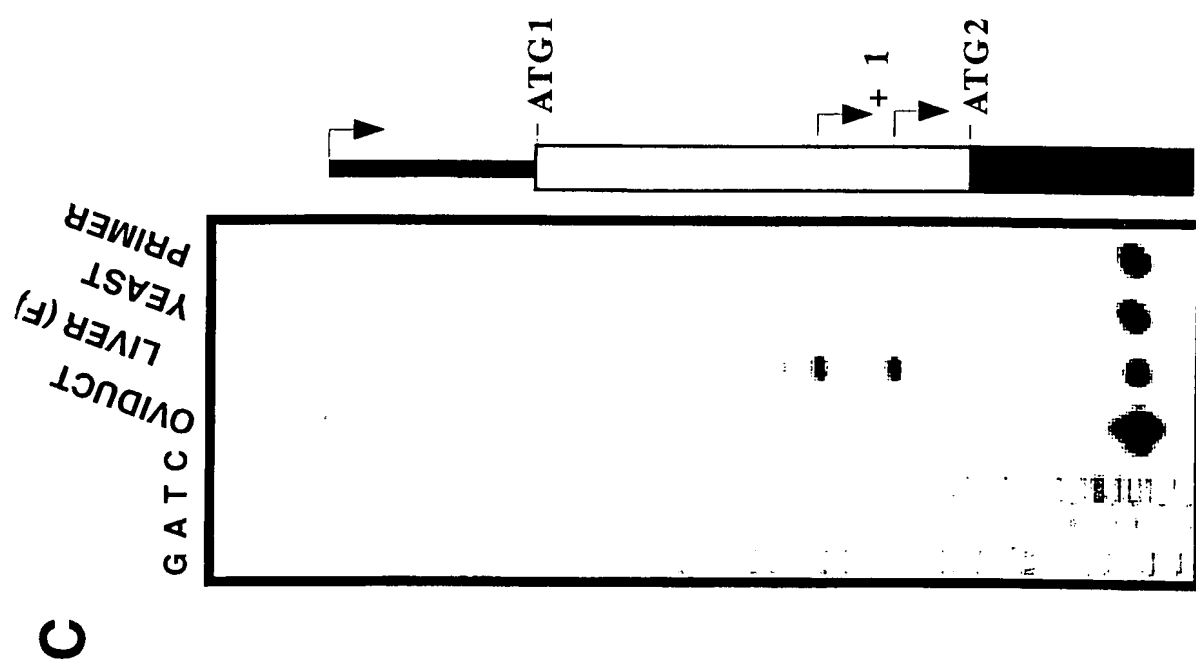


FIGURE 1D

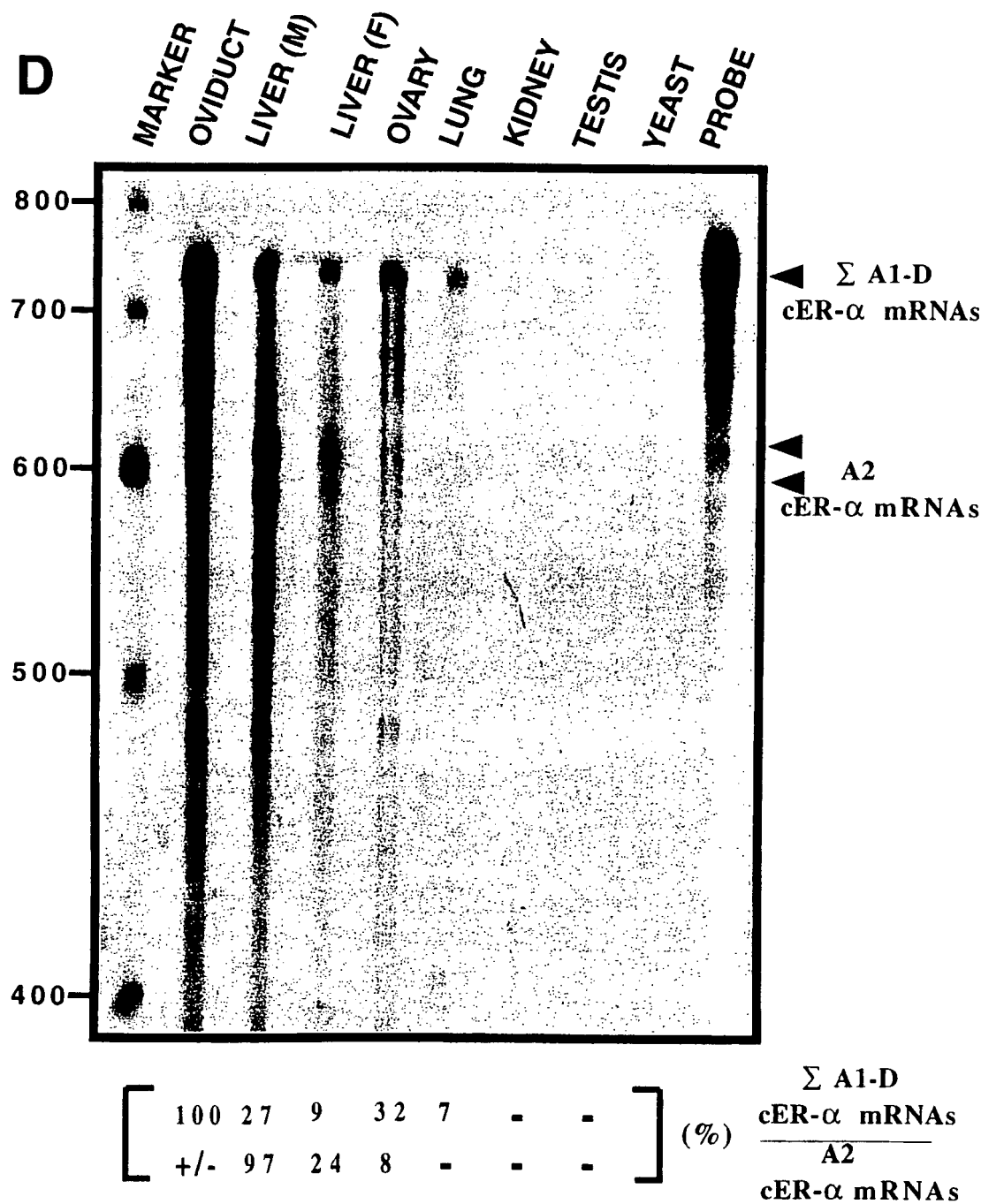


FIGURE 2A

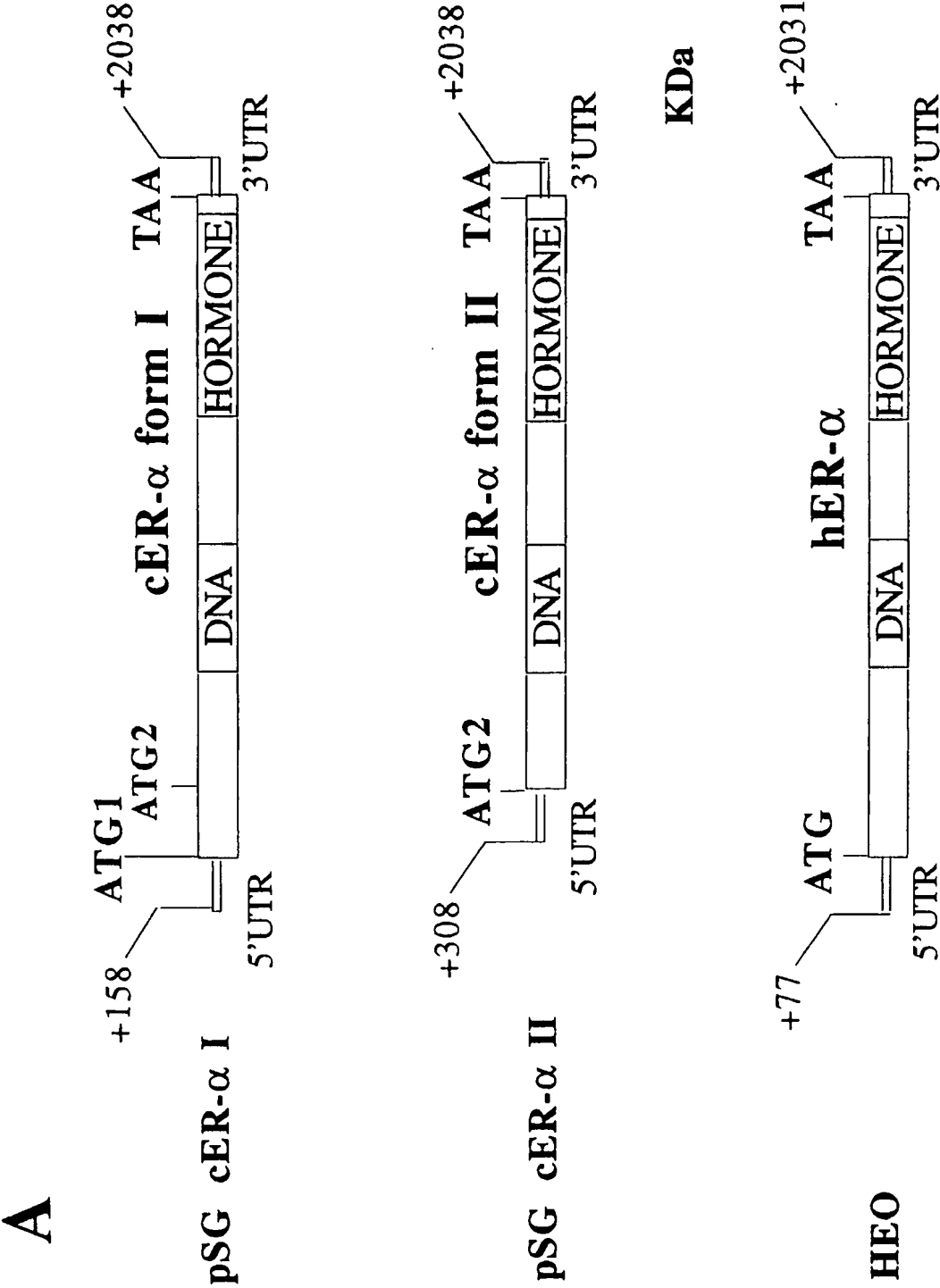


FIGURE 2B

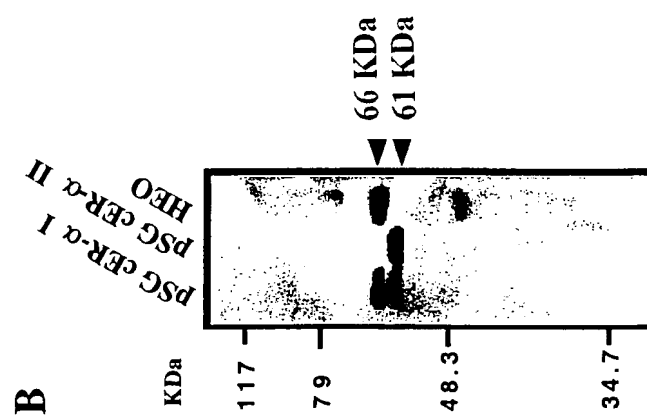


FIGURE 2C

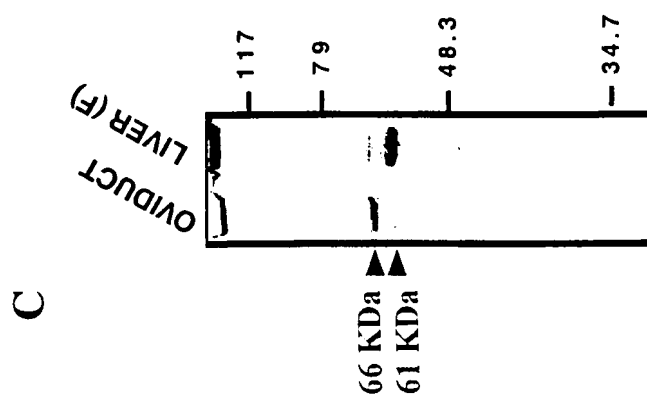


FIGURE 3A

A

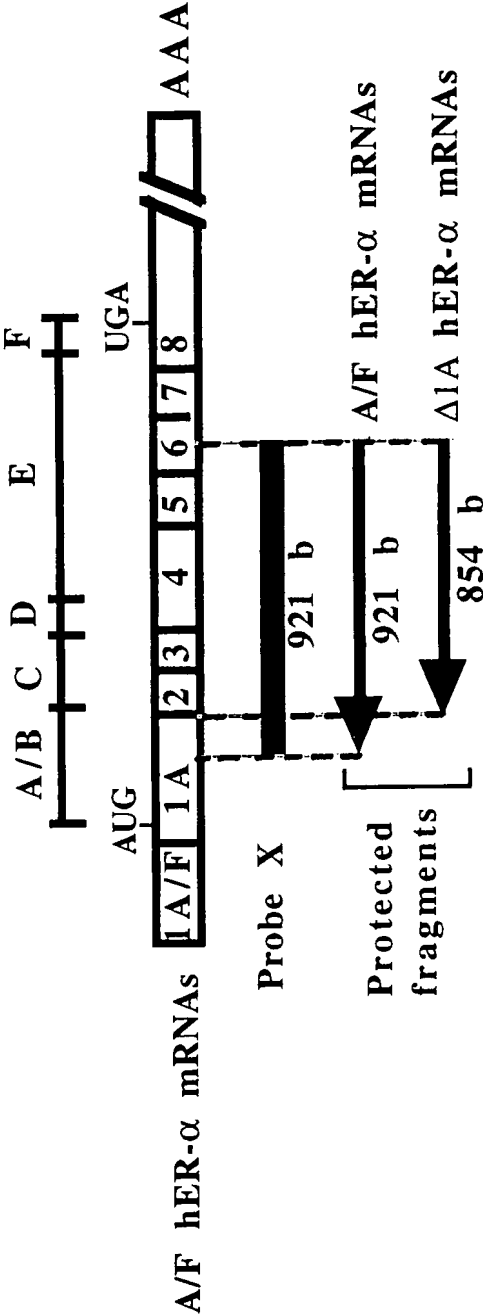


FIGURE 3B

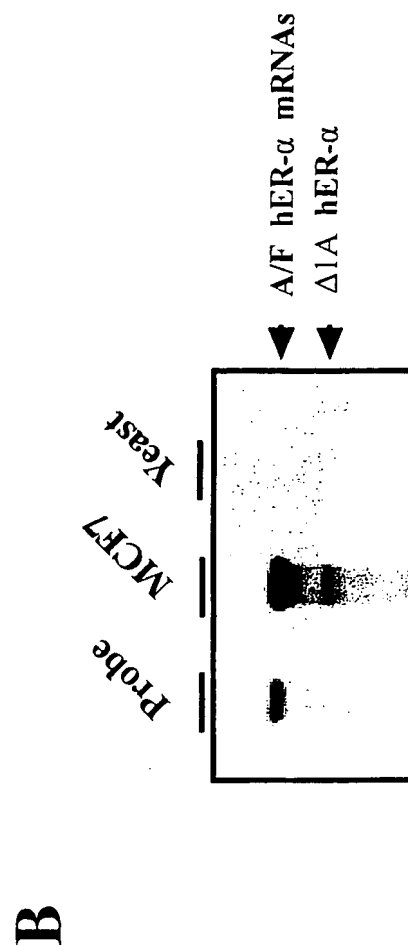


FIGURE 4A

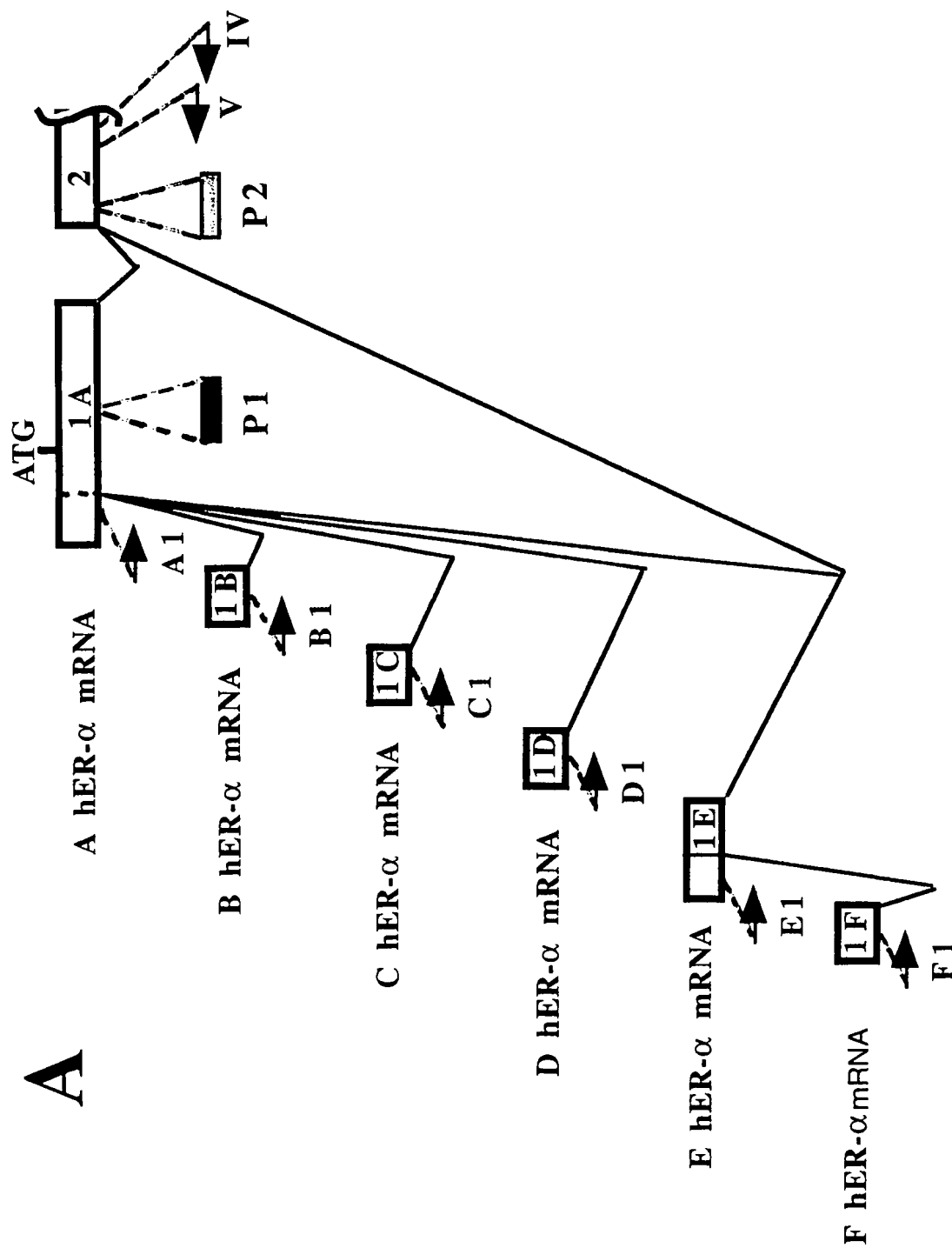


FIGURE 4B

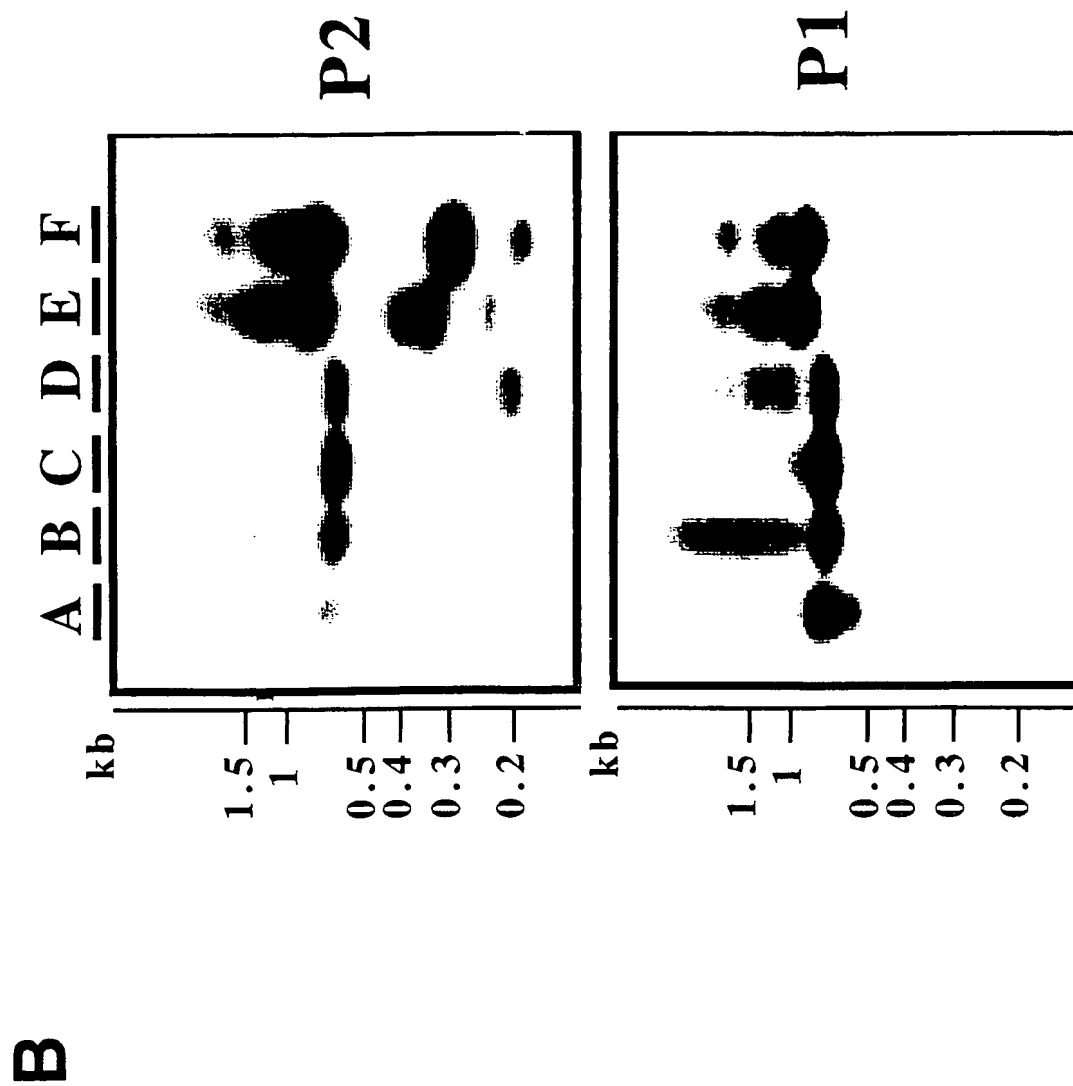
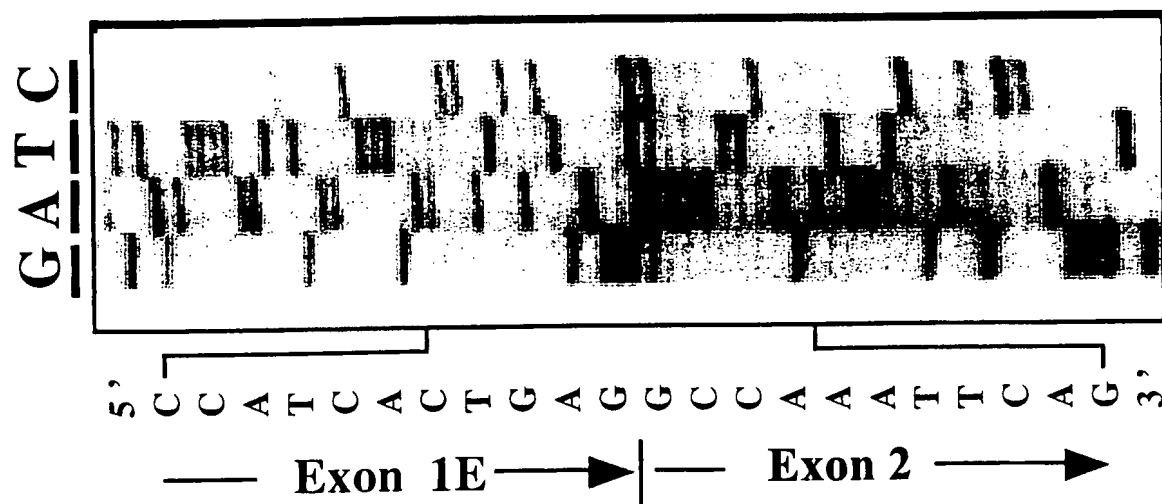


FIGURE 4C



C

FIGURE 5A

A

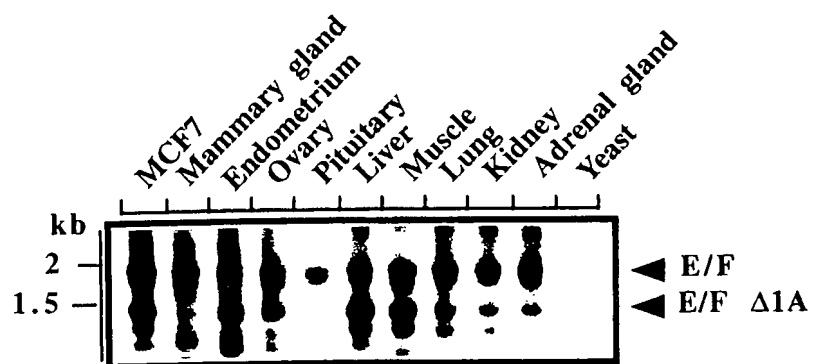
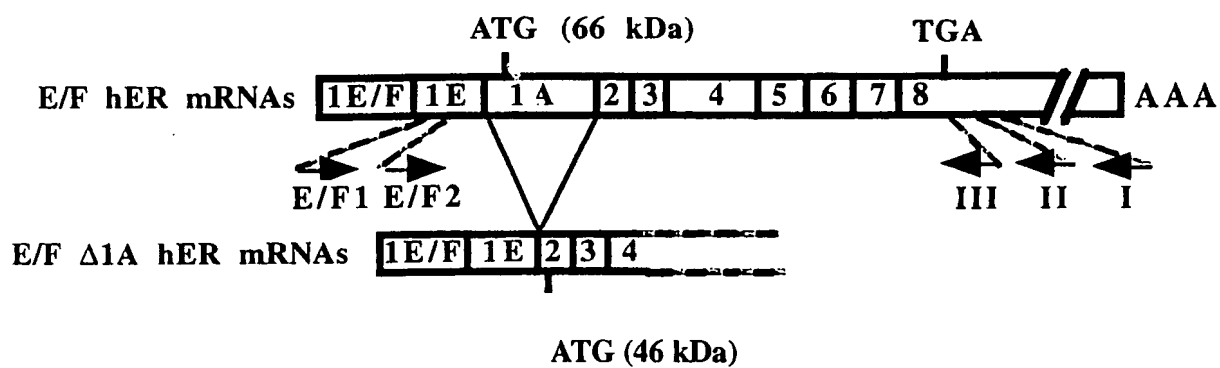


FIGURE 5B

B

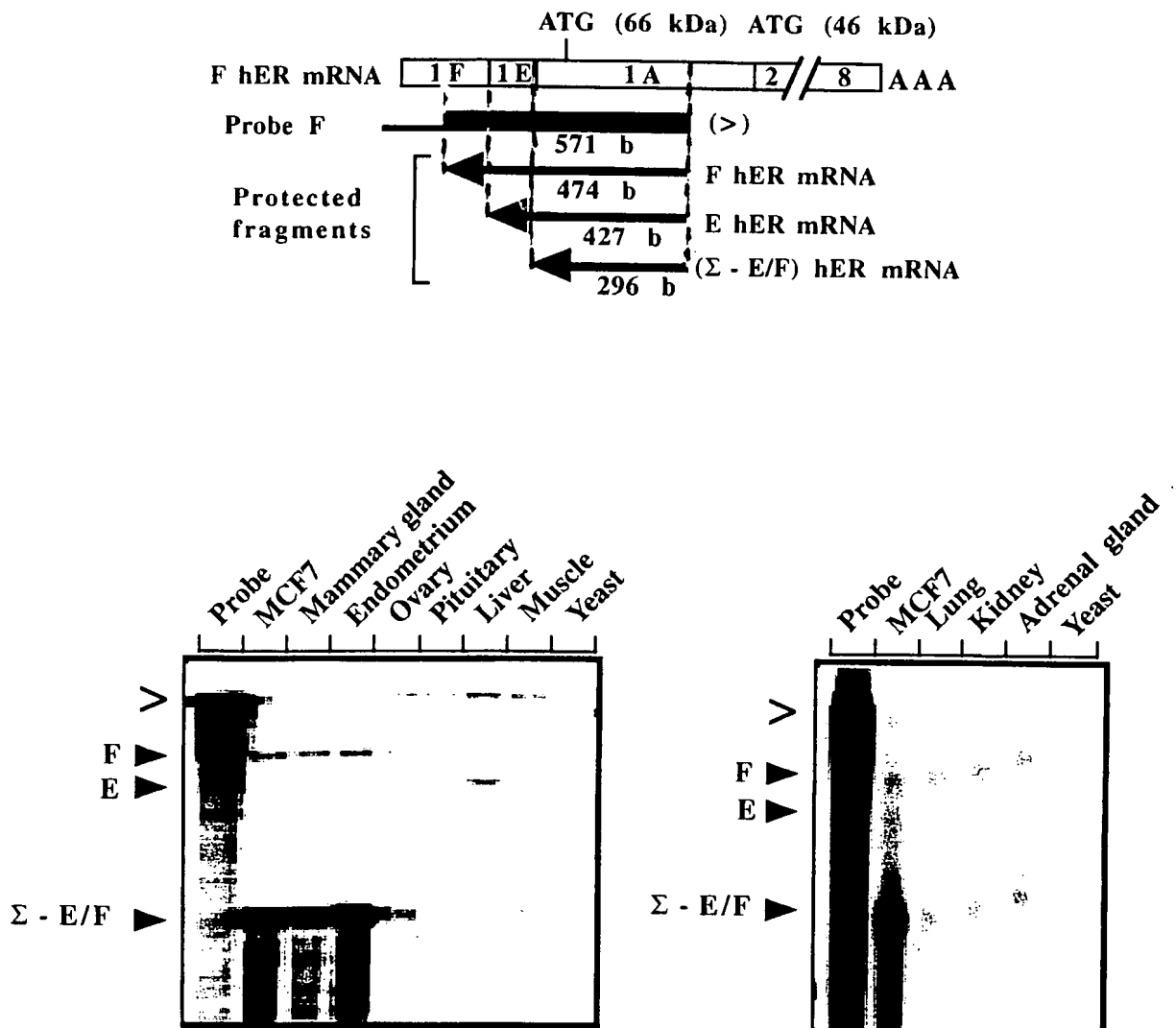


FIGURE 5C

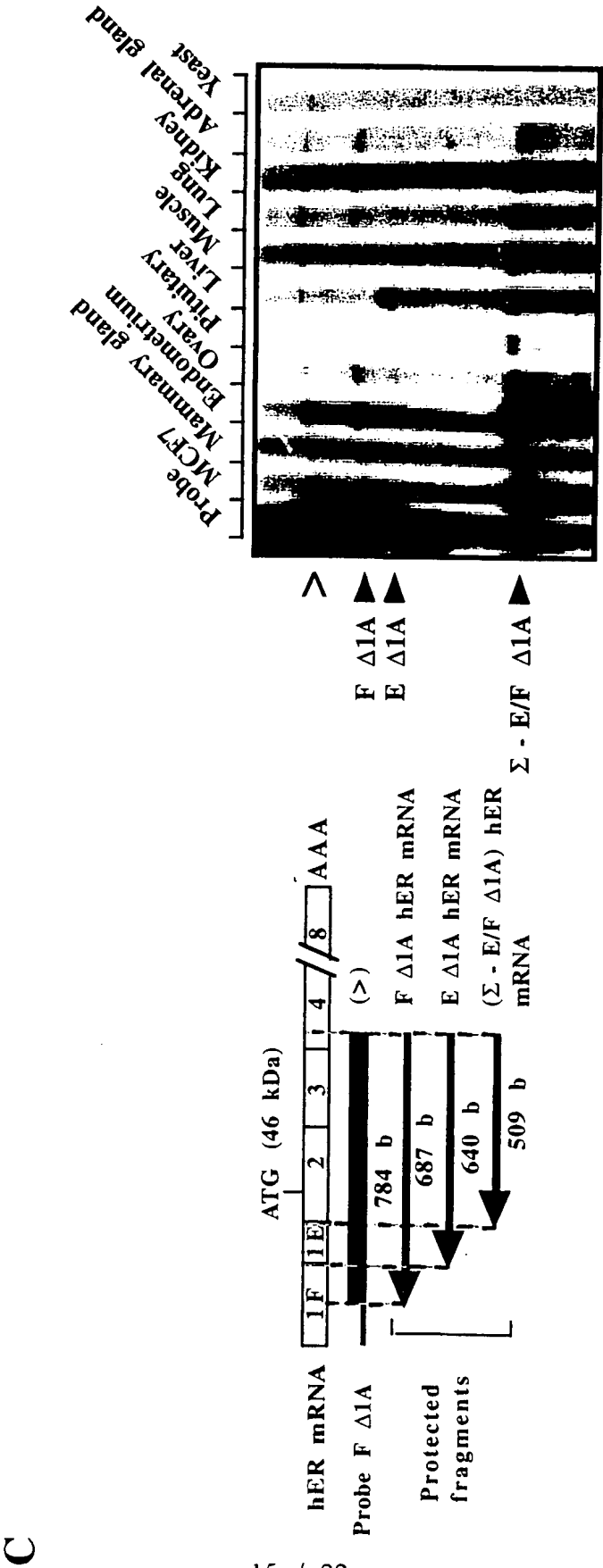
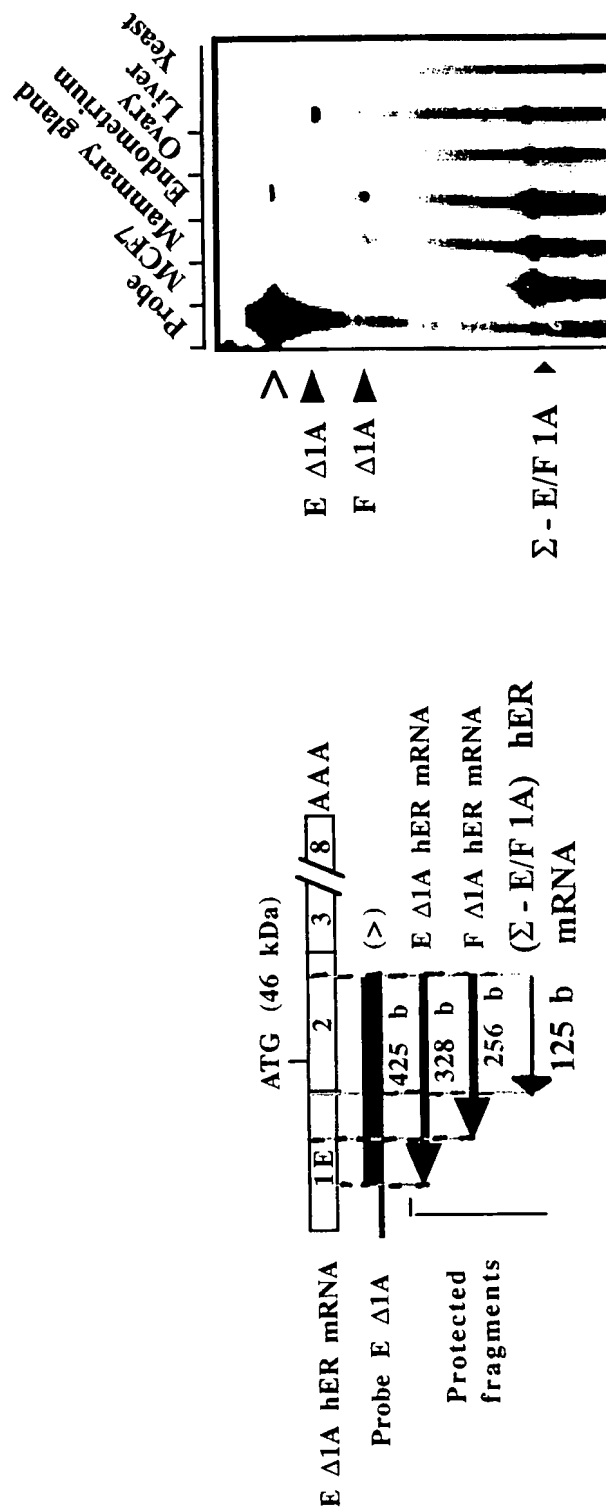


FIGURE 5D



D

FIGURE 6A

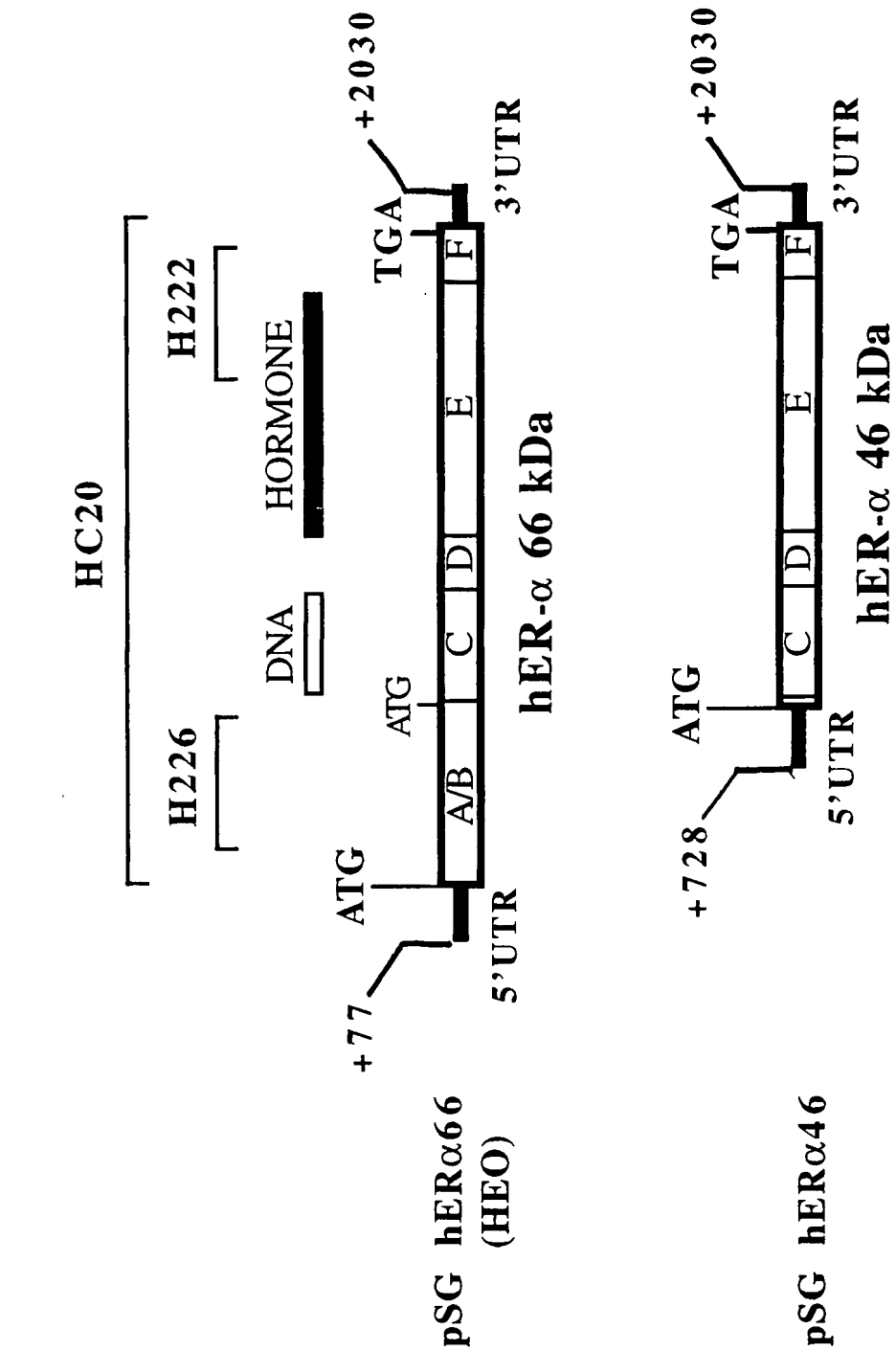


FIGURE 6B

B

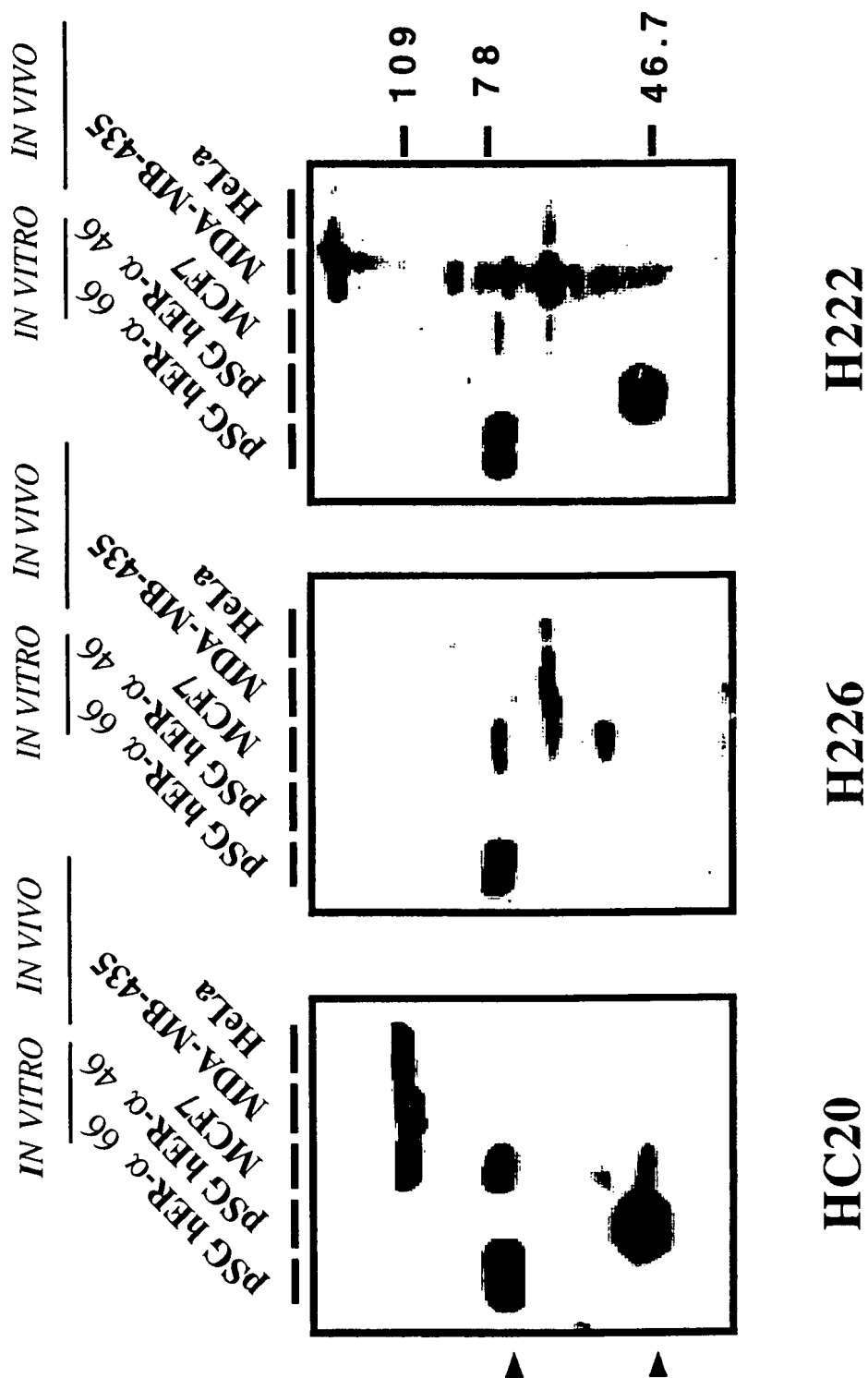


FIGURE 7

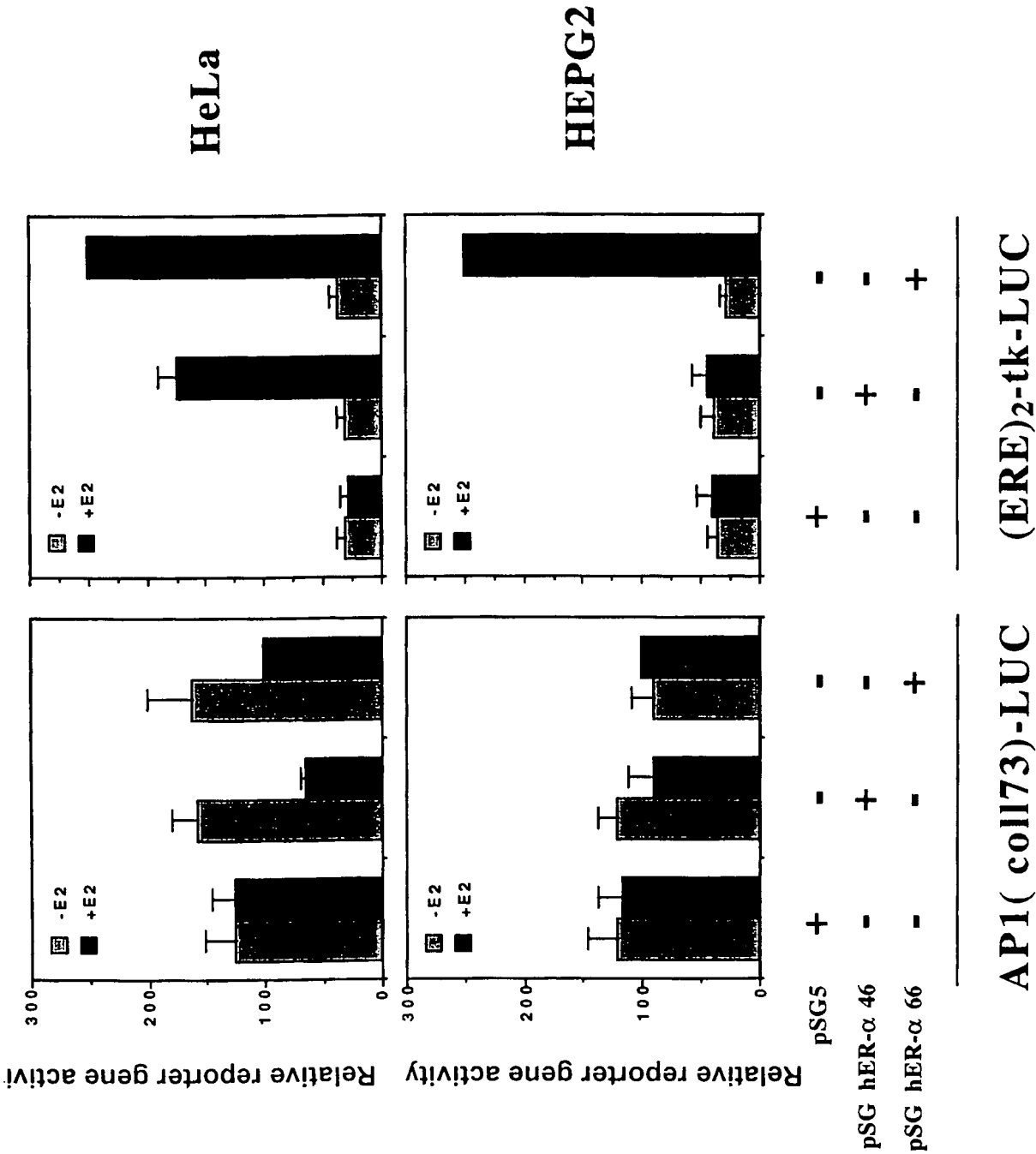
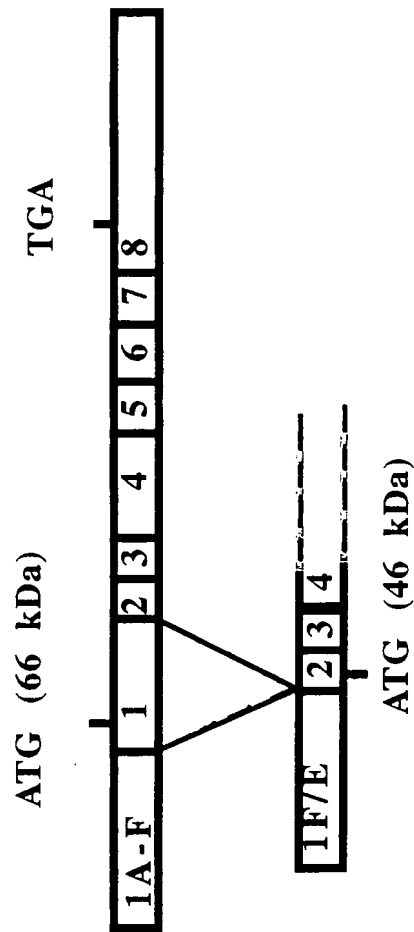
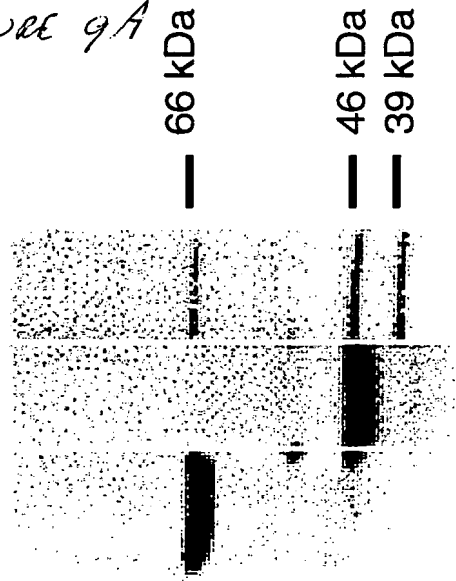


FIGURE 9A

osteoblasts

IVT 46

IVT 66



A

B

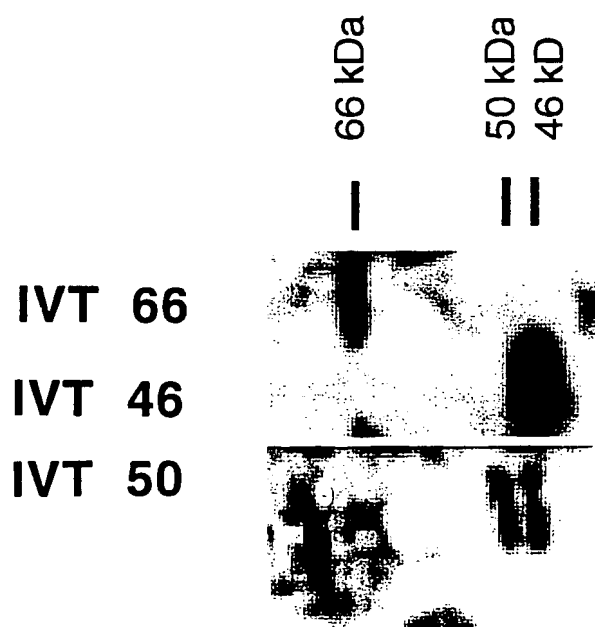
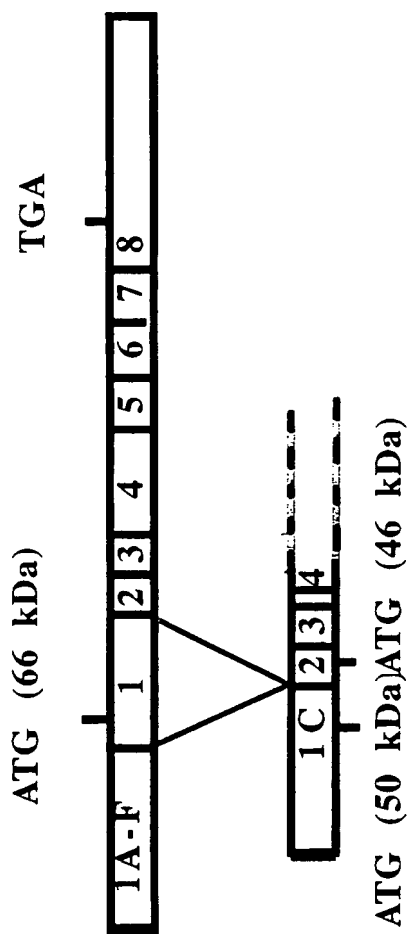


FIGURE 9B



SEQUENCE LISTING

<110> European Molecular Biology Laboratory

<120> Novel isoforms of the human estrogen receptor- α

<130>

<140>

<141>

<160> 18

<170> PatentIn Ver. 2.1

<210> 1

<211> 1223

<212> DNA

<213> umano

<400> 1

```

atgactatgc ttcaggctac cattatggag tctggtcctg tgagggctgc aaggccttct 60
tcaagagaag tattcaagga cataacgact atatgtgtcc agccaccaac cagtgcacca 120
ttgataaaaa caggaggaag agctgccagg cctgccggct ccgcaaagtc tacgaagtgg 180
gaatgatgaa aggtgggata cgaaaagacc gaagaggagg gagaatgttg aaacacaagc 240
gccagagaga tgatggggag ggcaggggtg aagtggggtc tgctggagac atgagagctg 300
ccaacctttg gccaaagccc ctcagatgca aacgctctaa gaagaacagc ctggccttgt 360
ccctgacggc cgaccagatg gtcagtgcct tgttggtatg tgagccccc atactctatt 420
ccgagtatga tcctaccaga cccttcagtg aagcttcgat gatgggctta ctgaccaacc 480
tggcagacag ggagctggtt cacatgatca actgggcgaa gagggtgcca ggctttgtgg 540
atctgaccct ccagatgacg gtccaccttc tagaatgtgc ctggctagag atcctgatga 600
ttggtctcgt ctggcgctcc atggagcacc cagtgaagct actgtttgct cctaacttgc 660
tcttgacag gaaccaggga aaatgtgtag agggcatggt ggagatcttc gacatgctgc 720
tggctacatc atctcggttc cgcagatgta atctgcaggg agaggagttt gtgtgcctca 780
aatctattat tttgcttaat tctggagtgt acacatttct gtccagcacc ctgaagtctc 840
tggaaagaaa ggaccatata caccgagtc tggacaagat cacagacact ttgatccacc 900
tgatggccaa ggcaggcctg accctgcagc agcagacca gcggctggcc cagctcctcc 960
tcacctcttc ccacatcagg cacatgagta acaaaggcat ggagcatctg tacagcatga 1020
agtgcagaa cgtggtgccc ctctatgacc tgctgctgga gatgctggac gccaccgcc 1080
tacatgcgcc cactagccgt ggaggggcat ccgtggagga gacggacca agccacttgg 1140
ccactgcggg ctctacttca tcgcattcct tgcaaaagta ttacatcacg ggggaggcag 1200
agggtttccc tgccacagtc tga
1223

```

<210> 2

<211> 1374

<212> DNA

<213> umano

<400> 2

```

atggaacatt tctggaaaga cgttcttgat ccagcagggc caaattcaga taatcgacgc 60
caggggtggca gagaaagatt ggccagtgacc aatgacaagg gaagtatggc tatggaatct 120
gccaaaggaga ctgcgtactg tgcagtgtgc aatgactatg cttcaggcta ccattatgga 180
gtctggtcct gtgagggtcg caaggccttc ttcaagagaa gtattcaagg acataacgac 240
tatatgtgtc cagccaccaa ccagtgcacc attgataaaa acaggaggaa gagctgccag 300
gcctgccggc tccgcaaag ctacgaagtg ggaatgatga aaggtgggat acgaaaagac 360
cgaagaggag ggagaatgtt gaaacacaag cgccagagag atgatgggga gggcaggggt 420
gaagtggggt ctgctggaga catgagagct gccaaccttt ggccaagccc gctcatgac 480
aaacgctcta agaagaacag cctggccttg tccctgacgg ccgaccagat ggctcagtgc 540

```

```

ttgttgatg ctgagcccc cataactctat tccgagtatg atcctaccag acccttcagt 600
gaagcttcga tgatgggctt actgaccaac ctggcagaca gggagctggg tcacatgata 660
aactgggcca agaggggtgcc aggcctttgtg gatttgaccc tccatgatca ggtccacctt 720
ctagaatgtg cctggctaga gatcctgatg attggtctcg tctggcgctc catggagcac 780
ccaggggaagc tactgtttgc tcctaacttg ctcttggaca ggaaccaggg aaaatgtgta 840
gagggcatgg tggagatctt cgacatgctg ctggctacat catctcggtt ccgcatgatg 900
aatctgcagg gagaggagtt tgtgtgcctc aaatctatta ttttgcttaa ttctggagtg 960
tacacatttc tgtccagcac cctgaagtct ctggaagaga aggaccatat ccaccgagtc 1020
ctggacaaga tcacagacac tttgatccac ctgatggcca aggcaggcct gaccctgcag 1080
cagcagcacc agcggctggc ccagctcctc ctcctcctct cccacatcag gcacatgagt 1140
aacaaaggca tggagcatct gtacagcatg aagtgcaaga acgtggtgcc cctctatgac 1200
ctgctgctgg agatgctgga cgcccaccgc ctacatgcgc ccactagccg tggaggggca 1260
tccgtggagg agacggacca aagccacttg gccactgcgg gctctacttc atcgcattec 1320
ttgcaaaagt attacatcac gggggaggga gaggggtttc ctgccacagt ctga 1374

```

<210> 3

<211> 422

<212> PRT

<213> umano

<400> 3

```

Met Ala Met Glu Ser Ala Lys Glu Thr Arg Tyr Cys Ala Val Cys Asn
  1                      5                      10                      15

```

```

Asp Tyr Ala Ser Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys
          20                      25                      30

```

```

Lys Ala Phe Phe Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Met Cys
          35                      40                      45

```

```

Pro Ala Thr Asn Gln Cys Thr Ile Asp Lys Asn Arg Arg Lys Ser Cys
          50                      55                      60

```

```

Gln Ala Cys Arg Leu Arg Lys Cys Tyr Glu Val Gly Met Met Lys Gly
          65                      70                      75                      80

```

```

Gly Ile Arg Lys Asp Arg Arg Gly Gly Arg Met Leu Lys His Lys Arg
          85                      90                      95

```

```

Gln Arg Asp Asp Gly Glu Gly Arg Gly Glu Val Gly Ser Ala Gly Asp
          100                      105                      110

```

```

Met Arg Ala Ala Asn Leu Trp Pro Ser Pro Leu Met Ile Lys Arg Ser
          115                      120                      125

```

```

Lys Lys Asn Ser Leu Ala Leu Ser Leu Thr Ala Asp Gln Met Val Ser
          130                      135                      140

```

```

Ala Leu Leu Asp Ala Glu Pro Pro Ile Leu Tyr Ser Glu Tyr Asp Pro
          145                      150                      155                      160

```

```

Thr Arg Pro Phe Ser Glu Ala Ser Met Met Gly Leu Leu Thr Asn Leu
          165                      170                      175

```

```

Ala Asp Arg Glu Leu Val His Met Ile Asn Trp Ala Lys Arg Val Pro
          180                      185                      190

```

```

Gly Phe Val Asp Leu Thr Leu His Asp Gln Val His Leu Leu Glu Cys
          195                      200                      205

```

Ala Trp Leu Glu Ile Leu Met Ile Gly Leu Val Trp Arg Ser Met Glu
 210 215 220
 His Pro Gly Lys Leu Leu Phe Ala Pro Asn Leu Leu Leu Asp Arg Asn
 225 230 235 240
 Gln Gly Lys Cys Val Glu Gly Met Val Glu Ile Phe Asp Met Leu Leu
 245 250 255
 Ala Thr Ser Ser Arg Phe Arg Met Met Asn Leu Gln Gly Glu Glu Phe
 260 265 270
 Val Cys Leu Lys Ser Ile Ile Leu Leu Asn Ser Gly Val Tyr Thr Phe
 275 280 285
 Leu Ser Ser Thr Leu Lys Ser Leu Glu Glu Lys Asp His Ile His Arg
 290 295 300
 Val Leu Asp Lys Ile Thr Asp Thr Leu Ile His Leu Met Ala Lys Ala
 305 310 315 320
 Gly Leu Thr Leu Gln Gln Gln His Gln Arg Leu Ala Gln Leu Leu Leu
 325 330 335
 Ile Leu Ser His Ile Arg His Met Ser Asn Lys Gly Met Glu His Leu
 340 345 350
 Tyr Ser Met Lys Cys Lys Asn Val Val Pro Leu Tyr Asp Leu Leu Leu
 355 360 365
 Glu Met Leu Asp Ala His Arg Leu His Ala Pro Thr Ser Arg Gly Gly
 370 375 380
 Ala Ser Val Glu Glu Thr Asp Gln Ser His Leu Ala Thr Ala Gly Ser
 385 390 395 400
 Thr Ser Ser His Ser Leu Gln Lys Tyr Tyr Ile Thr Gly Glu Ala Glu
 405 410 415
 Gly Phe Pro Ala Thr Val
 420

<210> 4
 <211> 457
 <212> PRT
 <213> umano

<400> 4
 Met Glu His Phe Trp Lys Asp Val Leu Asp Pro Ala Gly Pro Asn Ser
 1 5 10 15
 Asp Asn Arg Arg Gln Gly Gly Arg Glu Arg Leu Ala Ser Thr Asn Asp
 20 25 30
 Lys Gly Ser Met Ala Met Glu Ser Ala Lys Glu Thr Arg Tyr Cys Ala
 35 40 45

Val	Cys	Asn	Asp	Tyr	Ala	Ser	Gly	Tyr	His	Tyr	Gly	Val	Trp	Ser	Cys		
	50					55					60						
Glu	Gly	Cys	Lys	Ala	Phe	Phe	Lys	Arg	Ser	Ile	Gln	Gly	His	Asn	Asp		
65					70					75					80		
Tyr	Met	Cys	Pro	Ala	Thr	Asn	Gln	Cys	Thr	Ile	Asp	Lys	Asn	Arg	Arg		
				85					90					95			
Lys	Ser	Cys	Gln	Ala	Cys	Arg	Leu	Arg	Lys	Cys	Tyr	Glu	Val	Gly	Met		
			100					105					110				
Met	Lys	Gly	Gly	Ile	Arg	Lys	Asp	Arg	Arg	Gly	Gly	Arg	Met	Leu	Lys		
		115					120					125					
His	Lys	Arg	Gln	Arg	Asp	Asp	Gly	Glu	Gly	Arg	Gly	Glu	Val	Gly	Ser		
	130					135					140						
Ala	Gly	Asp	Met	Arg	Ala	Ala	Asn	Leu	Trp	Pro	Ser	Pro	Leu	Met	Ile		
145					150					155					160		
Lys	Arg	Ser	Lys	Lys	Asn	Ser	Leu	Ala	Leu	Ser	Leu	Thr	Ala	Asp	Gln		
				165					170					175			
Met	Val	Ser	Ala	Leu	Leu	Asp	Ala	Glu	Pro	Pro	Ile	Leu	Tyr	Ser	Glu		
			180					185					190				
Tyr	Asp	Pro	Thr	Arg	Pro	Phe	Ser	Glu	Ala	Ser	Met	Met	Gly	Leu	Leu		
	195						200					205					
Thr	Asn	Leu	Ala	Asp	Arg	Glu	Leu	Val	His	Met	Ile	Asn	Trp	Ala	Lys		
	210					215					220						
Arg	Val	Pro	Gly	Phe	Val	Asp	Leu	Thr	Leu	His	Asp	Gln	Val	His	Leu		
225					230					235					240		
Leu	Glu	Cys	Ala	Trp	Leu	Glu	Ile	Leu	Met	Ile	Gly	Leu	Val	Trp	Arg		
			245						250					255			
Ser	Met	Glu	His	Pro	Gly	Lys	Leu	Leu	Phe	Ala	Pro	Asn	Leu	Leu	Leu		
			260					265					270				
Asp	Arg	Asn	Gln	Gly	Lys	Cys	Val	Glu	Gly	Met	Val	Glu	Ile	Phe	Asp		
		275					280					285					
Met	Leu	Leu	Ala	Thr	Ser	Ser	Arg	Phe	Arg	Met	Met	Asn	Leu	Gln	Gly		
	290					295					300						
Glu	Glu	Phe	Val	Cys	Leu	Lys	Ser	Ile	Ile	Leu	Leu	Asn	Ser	Gly	Val		
305					310					315					320		
Tyr	Thr	Phe	Leu	Ser	Ser	Thr	Leu	Lys	Ser	Leu	Glu	Glu	Lys	Asp	His		
				325					330					335			
Ile	His	Arg	Val	Leu	Asp	Lys	Ile	Thr	Asp	Thr	Leu	Ile	His	Leu	Met		
			340					345					350				
Ala	Lys	Ala	Gly	Leu	Thr	Leu	Gln	Gln	Gln	His	Gln	Arg	Leu	Ala	Gln		
		355					360					365					
Leu	Leu	Leu	Ile	Leu	Ser	His	Ile	Arg	His	Met	Ser	Asn	Lys	Gly	Met		

370

375

380

Glu His Leu Tyr Ser Met Lys Cys Lys Asn Val Val Pro Leu Tyr Asp
 385 390 395 400

Leu Leu Leu Glu Met Leu Asp Ala His Arg Leu His Ala Pro Thr Ser
 405 410 415

Arg Gly Gly Ala Ser Val Glu Glu Thr Asp Gln Ser His Leu Ala Thr
 420 425 430

Ala Gly Ser Thr Ser Ser His Ser Leu Gln Lys Tyr Tyr Ile Thr Gly
 435 440 445

Glu Ala Glu Gly Phe Pro Ala Thr Val
 450 455

<210> 5
 <211> 20
 <212> DNA
 <213> umano

<400> 5
 ctcgcgtgtc ggcgggacat 20

<210> 6
 <211> 20
 <212> DNA
 <213> umano

<400> 6
 gctgcgtcgc ctctaacctc 20

<210> 7
 <211> 20
 <212> DNA
 <213> umano

<400> 7
 ctggccgtga aactcagcct 20

<210> 8
 <211> 20
 <212> DNA
 <213> umano

<400> 8
 atccagcagc gacgacaagt 20

<210> 9
 <211> 20
 <212> DNA
 <213> umano

<400> 9
tctctcggcc cttgacttct 20

<210> 10
<211> 21
<212> DNA
<213> umano

<400> 10
caagcccatg gaacatttct g 21

<210> 11
<211> 23
<212> DNA
<213> umano

<400> 11
agcctcaa atctccaaaa tct 23

<210> 12
<211> 27
<212> DNA
<213> umano

<400> 12
aattatattc tgtagctacg aaagaag 27

<210> 13
<211> 21
<212> DNA
<213> umano

<400> 13
ttctatagca taagaagaca g 21

<210> 14
<211> 14
<212> DNA
<213> umano

<400> 14
gagtgataat cttc 14

<210> 15
<211> 21
<212> DNA
<213> umano

<400> 15
attatctgaa ccgtgtggga g 21

<210> 16
<211> 21

<212> DNA
<213> umano

<400> 16
cgtgaagtac gacatgtcta c 21

<210> 17
<211> 47
<212> DNA
<213> umano

<400> 17
tctctcggcc cttgacttct gccaaattca gataatcgac gccaggg 47

<210> 18
<211> 49
<212> DNA
<213> umano

<400> 18
ccaccctggc gtcgattatc tgaatttggc agaagtcaag ggccgagag 49

INTERNATIONAL SEARCH REPORT

Internal Application No

PCT/EP 00/05981

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C07K14/72 A61K38/17 C07K16/28 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BARRAILLE, P. ET AL.: "Alternative initiation of translation accounts for a 67/45 kDa dimorphism of the human estrogen receptor ER.alpha." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 257, 2 April 1999 (1999-04-02), pages 84-88, XP002152550 the whole document	1-10
X	WO 87 05049 A (CALIFORNIA BIOTECHNOLOGY INC) 27 August 1987 (1987-08-27) the whole document	2,5-10



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *8* document member of the same patent family

Date of the actual completion of the international search

10 November 2000

Date of mailing of the international search report

28/11/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Smalt, R

INTERNATIONAL SEARCH REPORT

Internat. Application No

PCT/EP 00/05981

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MITCHNER NATASHA A ET AL: "Differential regulation and action of estrogen receptors alpha and beta in GH3 cells." ENDOCRINOLOGY, vol. 140, no. 6, June 1999 (1999-06), pages 2651-2658, XP000960607 ISSN: 0013-7227 the whole document ----	
A	WO 99 12961 A (MERCK & CO INC ;WILKINSON HILARY (US)) 18 March 1999 (1999-03-18) the whole document ----	
A	CLARET FRANCOIS-XAVIER ET AL: "Two functional forms of the Xenopus laevis estrogen receptor translated from a single mRNA species." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 19, 1994, pages 14047-14055, XP002152552 ISSN: 0021-9258 the whole document ----	
T	GRIFFIN, C. ET AL.: "Two functionally different protein isoforms are produced from the chicken estrogen receptor-alpha gene." MOLECULAR ENDOCRINOLOGY, vol. 13, no. 9, September 1999 (1999-09), pages 1571-87, XP000953442 the whole document ----	
T	FLOURIOT, G. ET AL.: "Identification of a new isoform of the human estrogen receptor-alpha (hER.alpha.) that is encoded by distinct transcripts and that is able to repress hER.alpha. activation function 1." EMBO JOURNAL, vol. 19, no. 17, 1 September 2000 (2000-09-01), XP002152553 the whole document -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 00/05981

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 8705049 A	27-08-1987	AU 7084787 A	09-09-1987
		EP 0258401 A	09-03-1988
		JP 63502397 T	14-09-1988
WO 9912961 A	18-03-1999	AU 9305298 A	29-03-1999
		EP 1012177 A	28-06-2000